Ecotoxicology of Copper Oxide Nanomaterials on the pond snail, *Lymnaea stagnalis*

by Valentina Ricottone

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Institute of Life and Earth Sciences,
School of Energy, Geosciences, Infrastructure and Society,
Heriot-Watt University
Edinburgh, United Kingdom

Abstract

Copper oxide nanomaterials (CuO NMs) are frequently employed for their antimicrobial properties in antifouling paints and other applications. Their extensive use can lead to contamination of aquatic ecosystems. In this research study, the freshwater snail, *Lymnaea stagnalis*, was used, as representative species, to identify biological and environmental impacts of CuO NMs on the benthic ecosystem.

An holistic evaluation of the toxicity of Cu as Cu^{2+} and CuO NMs (as pristine and safer by design (SbyD) CuO NMs (polyvinylpyrrolidone (PVP) and ascorbate (ASC)) and CuO Fragmented Products (FP)) was conducted, via acute and chronic exposure using different life stages of *L. stagnalis* and biological endpoints (mortality, reproduction and behaviour studies as well as molecular response).

Overall outputs demonstrated the higher sensitivity of juveniles compared to adults exposed to either ionic Cu or CuO NMs. Furthermore, data showed, in general, a higher toxicity of Cu ions compared to CuO NMs, despite equal concentration of dissolved Cu ions in solution, except for SbyD CuO-PVP functionalized in phosphate buffer.

Indeed, this project demonstrated that manufacturing conditions, dispersants used and medium, have an important role in determining fate and hazard of surface coatings of NMs. Furthermore, findings showed that acute experiments results are not always reflected when chronic experiments are conducted, since the fate of the individual NMs will be different, and hazard ranking of materials might differ across different exposure types and timelines. Moreover, the non-lethal toxicity, as well as the absence of any molecular response due to exposure to CuO_FP found in this study, highlights the need for more studies assessing the risk of forms of NMs more representatives of real exposure scenarios, such as NMs embedded in their matrix, rather than solely their pristine form.

Finally, promising findings were gathered using long term memory (LTM) formation test as a non-invasive endpoint to assess CuO NMs toxicity on *L. stagnalis*, providing an early toxicity indication compared with the more conventional LC50 approach.

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List of abbreviations

4PL	4-parameters logistic
AFM	Atomic force microscopy
AFP	Aged fragmented product/s
Ag	Silver
ANOVA	Analysis of variance
ASC	Ascorbate
BWT	Biological wastewater treatment
CAT	Catalase
CeO_2	Cerium oxide
CNS	Central nervous system
CNTs	Carbon nanotubes
CPI	Consumer Product Inventory
CSA	Centrifugal separation analysis
CTR	Copper transport regulatory
CuO	Copper oxide
CuO NM/s	CuO nanomaterial/s
Cu	Copper
DLS	Dynamic light Scattering
DNA	Deoxyribonucleic acid
DOM	Dissolved organic matter
EC	Effective concentration
EHS	Environmental, health and safety
ELS	Electrophoretic light scattering
EPS	Extracellular polymeric substances
FP	Fragmented product
GPX	Glutathione peroxidase
GR	Glutathione reductase
HDD	Hydrodynamic diameter
HSP	Heat-shock proteins
ICP-OES	Inductively coupled plasma optical emission spectrometry
IS	Ionic strength
ISO	International Standards Organisation
ITM	Intermediate-term memory
JRC	Joint Research Centre
LC	Lethal concentration
LCA	Life-Cycle Assessment
LOEC	Lowest observed effect concentration
LTM	Long term memory
MP/s	Microparticle/s
Mt	Memory test
MT/s	Metallothionein/s
MWCNT/s	Multi wall carbon nanotube/s
MWM	Morris water maze

NOEC	No observed effect concentration
NOM	Natural organic matter
NM/s	Nanomaterial/s
NP/s	Nanoparticle/s
OECD	Organisation for Economic Co-operation and Development
PEC	Predicted environmental concentration
PCR	Polymerase chain reaction
PBS	Phosphate buffer saline
pH	Potential Hydrogen
PSD	Particle size distributions
PVP	Polyvinylpyrrolidone
QD	Quantum dots
RCF	Relative centrifugal force
RM/ANOVA	Repeated measurement/ analysis of variance
ROS	Reactive oxygen species
RT	Reverse transcription
SbyD	Safer by design
SEM/SE	Standard Error of the Mean
SOD	Superoxide dismutase
SUN	Sustainable Nanotechnologies project
SWCNTs	single wall carbon nanotubes
TEM	Transmission electron microscopy
TGA	Thermogravimetric analysis
TS	Training session
WFP	Weathered Fragmented Products
ZnO	Zinc oxide

Chapter 1 Introduction

Chapter 1 provides an overview of the research field addressed in this thesis. Initially a review of the key scientific aspects of nanotechnology, nanomaterials, their fate and behaviour in the environment and their potential toxicity to organisms is presented. Subsequently, the specific case of study of this research project, copper oxide nanomaterials (CuO NMs), is described, focussing on their fate in the aquatic ecosystem and their reported ecotoxicity to organisms. Characterisation results of these nanomaterials are also reported. Finally, the biology, behaviour and use in risk assessment studies of the model species pond snail *Lymnaea stagnalis* (Linnaeus, 1758*)*, are reviewed. In this research project, this snail was used as representative species of the benthic ecosystem, aiming to assess the potential toxicity of CuO NMs in this compartment of the aquatic environment. Finally, the aim and general objectives of the research project are clearly outlined.

1.1 Nanotechnology and Nanomaterials

Over the past decade, advancements in the fields of nanoscience and nanotechnology, due to the manufacture of nanoscale materials with novel physicochemical properties, have led to an increase in the use of nanomaterials (NMs) in a wide range of applications worldwide across engineering, medicine, agriculture, food industries and biotechnology (Kumar et al. 2018). Despite nanotechnology continuing to be a developing area, today NMs have been incorporated in products used in society's daily lives, showing that nanotechnology is well on its way to becoming a general-purpose technology with considerable economic impact (Roco et al. 2011).

Little information is still available on the real amount of consumer products that contain NMs (often called nano-enabled products), hindering a real qualitative and quantitative human, and environmental risk assessment (Foss Hansen et al. 2016). Currently, several databases containing information about the marketed NMs and nano-enabled products exist (*e.g.* Consumer Product Inventory (CPI)) Vance et al. (2015); however, they are often poorly updated and generally collecting data only at country levels (*e.g.* Danish nano-product registry). An exception is found in "The Nanodatabase", a European inventory of products containing NMs, updated directly by the producers (Foss Hansen et al. 2016), which as of January 2018, documented around 3000 nano-enabled products mainly being included in the Health and Fitness category. Silver NMs are reported to be the most used NMs, however about 60% of the product registered are classified as unknown, with the real amount used of different NMs not reported (Fig. 1-1) (Tænk 2013).

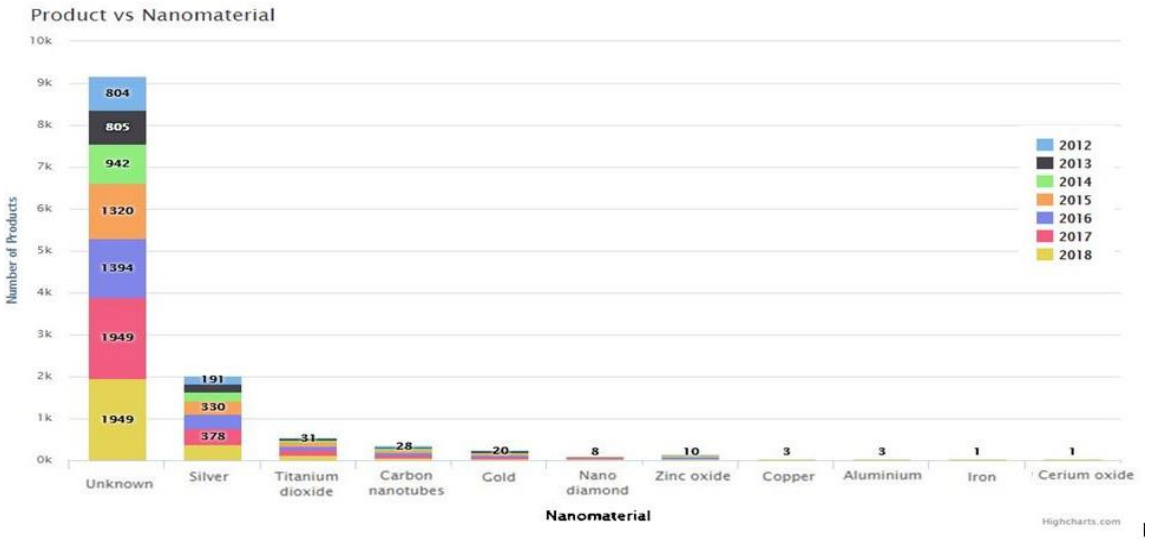

Figure 1-1 Analysis from the Nanodatabase website: number of nano-enabled products containing different NMs through 2012-2018. "Unknown nanomaterial" is used when it is not declared the exact form of NMs (Tænk 2013).

In 2011, the European Commission published a Recommendation (2011/696/EU) (EU 2011) with a proposed definition, for legislative and policy purposes, for the term "nanomaterial" based on the size of the particles of the material, without regard to specific functional or hazardous properties or risk (Rauscher et al. 2014). The definition states: *"'Nanomaterial' means a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm–100 nm. In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50% may be replaced by a threshold between 1% and 50%".* Furthermore, a material can be regarded as NM if the specific surface area by volume of the material is greater than 60 m^2cm^{-3} (Bleeker et al. 2013).

The EU Recommendation also considers as NMs, particles in agglomerates or aggregates whenever the constituent particles are in the size range 1 nm- 100 nm (Fig. 1-2), defining:

- 'particle' as a minute piece of matter with defined physical boundaries;
- 'agglomerate' as a collection of weakly bound particles or aggregates where the resulting external surface area is similar to the sum of the surface areas of the individual components;
- 'aggregate' as a particle comprising of strongly bound or fused particles. Agglomerated or aggregated particles may exhibit the same properties as the unbound particles.

In 2011, within the Recommendation, it was announced that the proposed definition would be reviewed in 2014, in light of possible new science discoveries that could lead to a modification of the definition itself. The review of the definition, started in 2014, was divided in three parts and concluded in 2015 with the publication of the last and third part of the review, "*Towards a review of the EC Recommendation for a definition of the term nanomaterial Part 3: scientific-technical evaluation of options to clarify the definition and to facilitate its implementation*" (Rauscher et al. 2015), which described scientific and technical options to clarify the wording and facilitate the implementation of the definition. The options presented in the report will be evaluated by the EC policy services, which will decide if ultimately the definition given in 2011 need to be revised or supported with additional guidance (Rauscher et al. 2017).

Simultaneously, in 2015 the International Standards Organisation (ISO) revised the technical specification on the terminology and definitions of nano-objects provided in ISO 2008, publishing a new report where nano-objects are defined as "*materials with one or more external dimensions in the nanoscale (1 nm to 100 nm), which can have properties that make them key components of materials and systems resulting in improved performance over their conventional counterparts. The size and shape of nano-objects are often intrinsic to their function, so the description and measurement of their size and shape are important and must be considered carefully*" (ISO 2015).

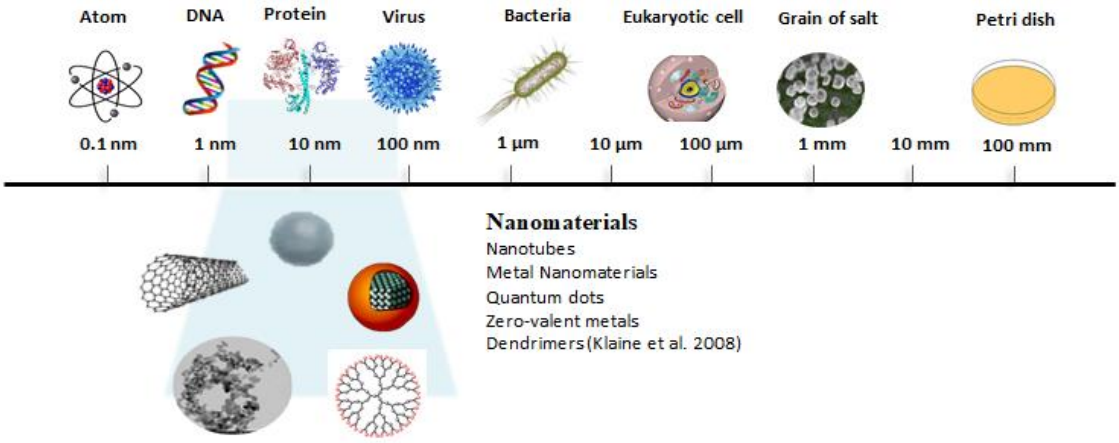

Figure 1-2 Relative positions of NMs on a size based scale diagram from 0.1 nm to 100 mm. Adapted from Klaine et al. (2008).

In the paucity of a global international conformity regarding the terminology of NMs, including the different definitions in use the EU across the different sectors (Rauscher et al. 2017), the above mentioned definition from JRC (EU 2011) will be preferred herein, indicating as NMs either nanoparticles (NPs), nano-objects and their aggregates or agglomerates.

NMs can occur in the environment naturally, forming within and distributed throughout soils, ground and surface waters, sediments, the oceans and the atmosphere (Hochella Jr et al. 2012); as well as artificially produced by a wide range of industrial physical and chemical procedures (*e.g.* milling or attrition, nucleation of molecular components) (Ju-Nam and Lead 2008), or produced incidentally, via combustion (Bleeker et al. 2013)

Klaine et al. (2008) categorized man made NMs in five classes: carbon nanotubes (CNTs) and related materials (single wall carbon nanotubes (SWCNTs), multi wall carbon nanotubes (MWCNTs), fullerenes, grapheme, etc.); metal containing materials, including metal oxides, transition metals, their compounds or composites (*e.g.* zinc oxide (ZnO), cerium oxide (CeO_2), copper oxide (CuO)); semiconductor nanocrystals namely quantum dots (QDs); zero-valent metals (*e.g.* zero-valent iron) and dendrimers, multifunctional polymers. Metal and carbon NMs are primarily used in a vast number of industrial applications (Kumar et al. 2012), such as commercial products, environmental applications, sustainable chemistry and health (Amde et al. 2017). Already in 2009, metal NMs represented in 30% of the market share of consumer nano-enabled products of which a considerable part are CuO NMs used in energy storage, sensors, surfactants, catalysts, and as antimicrobial agents in various industries, agricultural activities and environmental remediation (Peng et al. 2017).

At the nanoscale, materials have different or heightened physico-chemical properties (size, surface/volume ratio, shape, chemical composition) compared with their micro-size counterparts. This is in part due to a larger relative surface area that results in a modified surface reactivity (Bondarenko et al. 2013). Whereas, due to these unique physico-chemical properties nanotechnology has gathered increasing attention from the industry sector, some concerns have arisen with respect to potential health effects on humans and the environment (Fent 2010). In this perspective, due to increasing production volumes of man-made NMs and thus raising the possibility of occupational and environmental exposure to NMs by unintentional (or intentional) release (Bondarenko et al. 2013), many studies have begun to identify their potential risks to human health, ecosystems and organisms.

This research project was developed within the scope of Work-package 4, *"Ecotoxicology, Effects on Ecosystem Services and Ecological Risk"*, of the European Commission's FP7 project SUN "Sustainable Nanotechnologies" which aimed to address the entire lifecycle of NMs creating a risk assessment and decision support system for users of NMs as well as developing methods to reduce both adverse effects

and exposure to acceptable levels. In particular, this research project is focussed on the impact of CuO NMs in the benthic freshwater ecosystem.

1.1.1 Environmental fate and toxicity of NMs

Nanomaterials can be released in the environment during various points of the life cycle (production, use and disposal) of a nano-enabled product. Their particular physico-chemical properties can be affected by environmental parameters modifying their behaviour once they interact with biological systems (Fent 2010).

Nowack et al. (2012a) analysed the potential for exposure of the environment to NMs. The main sources of contamination were identified in the agro-food and textile sector, during ground water and soil remediation (Nowack et al. 2012a). NMs concentrations in the environment are forecast to be in the ngL^{-1} to μgL^{-1} range (Keller and Lazareva 2014), however different authors that have attempted to model and predict the concentration of NMs released in the environment (Gottschalk et al. 2013, Adeleye et al. 2014); ultimately, stated the uncertainty of the gathered data due the meagre amount of information on NMs production rates, disposal stages data, and release data available in literature (Keller and Lazareva 2014, Caballero-Guzman and Nowack 2016).

Furthermore, it has been emphasized that it is unfeasible to assess the environmental risks of NMs by only evaluating the possible fate and toxicity of the pristine NMs (Lowry et al. 2012, Mitrano et al. 2015, Sun et al. 2016). When NMs are released from products, they usually do not exist as loose NMs, but they are more likely to be embedded in a matrix and exposed to various abiotic and biotic factors, which would likely affect their physico-chemical proprieties and consequently their eventual toxicity. Thus, data related to pristine NMs may not always be representative of real-world exposure scenarios of the released materials (Kuhlbusch and Nickel 2010, Mitrano and Nowack 2017).

This is especially important for metal-based NMs, where, in the aquatic environment, depending on the proprieties and chemical composition of the receiving environment, the base metals themselves can persist and might occur in different compounds or phases which affect metal bioavailability (Wang et al. 2013).

Peijnenburg et al. (2015) highlighted the key factors that might play a major role in governing the fate of NMs in an aquatic environment. These include partitioning between sedimented and suspended forms, and degradation by chemical (*e.g.* pH), physical (*e.g.* sunlight), and/or biological means (biotransformation). Therefore, depending on parameters such as temperature, ionic strength, pH, water hardness, NM

concentration and size NMs may undergo multiple transformation processes that might also take place at the same time or in sequence, such as agglomeration, aggregation, degradation, solubilisation, sorption, and/or sedimentation (Handy et al. 2008, Mitrano and Nowack 2017).

In general, aggregation, agglomeration and deposition are connected processes. In this thesis, the term agglomeration will be used to represent both processes of agglomeration and aggregation, unless reporting directly from studies, where the respective authors' terminology will be used.

Deposition of NMs, determined by their properties and a combination of physicochemical parameters of the exposure medium, increases when agglomeration is high (Amde et al. 2017). Faster agglomeration and sedimentation indicate that NMs will not remain in the water column for long (hours to days) and thus pelagic aquatic species exposure may be limited with increased and prolonged exposure to benthic species. Slower sedimentation (weeks) indicates that NMs will be transported over greater distances, but it may also mean greater dilution over time (Garner and Keller 2014). Areas near points of release (*e.g.* wastewater effluent discharge) may show higher NM concentrations in the benthic system over time, and may need to be monitored carefully for environmental impacts. For instance, Stegemeier et al. (2017) investigated the rate of transformation of Cu and Ag NMs (CuO, CuS, Ag^0 and Ag_2S NMs and soluble $CuNO_3$) in a long-term (9 months) realistic environment, using a freshwater wetland mesocosm approach. Results indicated, at the high concentrations of NMs tested (100 mg kg^{-1} in the top layer of the sediment), a fast transformation of the deposited NMs into the sediment. Within 1 week, after introduction of the NMs, only < 4% of Ag of the Ag NMs was detected as Ag^0, the remainder was detected as silver sulphide and a minor part as Ag bound to thiol species present. In contrast, no pristine CuO or CuS NMs remained in the superficial sediment and Cu was mostly found bound to sulphides or organic matter regardless of the initial form of Cu NMs added. Furthermore, the formation of elemental Cu^0 was observed in the mesocosm treated with either form of Cu NMs but, surprisingly, no in the $Cu(NO)_3$, stressing a difference in the availability and distribution of Cu in the two forms. Finally, after 9 months of exposure, ~50 ± 15% of the total silver and copper added as NMs was taken up into the plant tissues of *E. densa* (Stegemeier et al. 2017).

Other studies indicate that the presence of dissolved organic matter (DOM) influences the transport and fate of NMs. This includes covering the surface of the NMs due steric or electrostatic repulsion (Bundschuh et al. 2016) thus modifying their

aggregation/agglomeration behaviour (Adeleye et al. 2014, Mohd Omar et al. 2014, Conway et al. 2015, Keller et al. 2017). Mohd Omar et al. (2014) conducted a study on the aggregation and disaggregation of ZnO NMs over a wide range of pH, at different concentrations of humic acid, and found that the anionic charges carried by aquatic humic substances played a major role in the aggregation and disaggregation of ZnO NMs. With increasing concentration of humic acid (0.1-0.5 mgL^{-1}) and below the isoelectric point (pH_{PZC}), the anionic humid acid was rapidly adsorbed onto the positively charged ZnO NMs promoting aggregation. In contrast, when close to the pH_{PZC}, the humic acids promoted partial disaggregation controlling the suspension behaviour of the NMs. Finally, when pH was greater than pH_{PZC} the humic acid formed a surface coating on the ZnO NMs enhancing stability via electrostatic and steric interactions (Mohd Omar et al. 2014).

The coating of metal NMs in the environment by DOM may also reduce their dissolution into the water phase (Bundschuh et al. 2016) affecting their fate, toxicity, persistence and bioavailability. Like the other processes, dissolution also depends on the NMs physicochemical properties and chemistry of the environmental system (Amde et al. 2017). Consequently, NMs released into different types of aquatic environments (*e.g.* varying hardness, salinity and redox potential) are expected to behave differently (Handy et al. 2008, Peijnenburg et al. 2015).

Findings from fate and hazard studies of NMs report different, and sometimes conflicting, results. Frequently there is a strong correlation between increased toxicity and degree of dissolution of the NMs in the exposure environment. Indeed, toxicity of metal NMs has been attributed to free metal ion concentration leached from the NMs (Heinlaan et al. 2008, Griffitt et al. 2008, Heinlaan et al. 2011), to endocytosis mediated NMs' response (Pradhan et al. 2012), to a complex interaction of both mechanisms (Misra et al. 2012b) or to possible synergistic effects with other chemicals already present in the environment (Henry et al. 2013).

In the literature, there is lack of clarity about the definition of the last two aforementioned processes, which are both defined as 'Trojan horse' effect. This definition was first proposed by Limbach et al. (2007) describing the role of Co_3O_4 NMs in enabling the transport of high levels of cobalt into the human lung epithelial cells, that otherwise as ionic Co would hardly enter the intact cell membranes. This concept was then extended to the capacity of NMs to bind and carry on their surfaces other chemicals (including pollutants) with them, as they move through environmental and biological systems, modifying ultimately NMs toxicity and biological availability

(Baun et al. 2008b). Nowadays both definitions are still used to describe a NMs Trojan horse effect.

For instance, Gunawan et al. (2011) found that the inhibition of growth of *Escherichia coli* was caused by the leaching of ions from CuO NMs in the amino acid-rich medium leading to formation of copper-peptide complexes, rather than by the NMs themselves, indicating this as Trojan horse effect. These complexes induced a multiple fold increase in intracellular reactive oxygen species (ROS) generation and reduced the fraction of viable cells, resulting in the overall inhibition of biomass growth (Gunawan et al. 2011).

Differently, Park et al. (2010) followed the original definition describing the citoxicity of Ag NMs to rat macrophage cells by ionization of the NMs once they entered the cell. In fact, after exposure, Ag NMs were observed in the cytosol of the activated cells and the culture medium but were not observed in the dead cells suggesting a Trojan horse mechanisms (Park et al. 2010).

In this research project, the original definition of Trojan horse effect of Limbach et al. (2007) will be used.

An emergent amount of studies on the toxicity of NMs to different freshwater or marine species have been conducted mostly at a range of concentrations not always mirroring the predicted environmental concentrations, using various measures of toxicity (not always comparable), such as: no observed effect concentration (NOEC), lowest observed effect concentration (LOEC), median lethal dose (LD50), median lethal concentration (LC50), half maximal effective concentration (EC50) (Garner and Keller 2014). However, to understand the risk of NMs in the environment, concentration response effects as well as the exposure pathway of NMs should be determined. Factors that may influence toxicity and uptake into an organism include NM size, charge, surface area, and shape. Toxicity will also depend on the persistence of NMs within the organism. NMs can enter an organism via the gastrointestinal tract through water, food, cosmetics, drugs, and medicines (Garner and Keller 2014), as well as through the respiratory surfaces or gills (Felix et al. 2017) and skin or body surface (Platten et al. 2016, van Pomeren et al. 2017). Toxicity of NMs might cause either mechanical damage (*e.g.* blockage of gills, coating of the organisms) or a physiological response (*e.g*. ROS production).

Baumann et al. (2014) found that exposure to coated iron oxide NMs caused the moulting inhibition of neonates of *Daphnia magna* due the adsorption of the NMs to the carapace, especially close to the filtering apparatus and the gills, hindering a complete ecdysis (moulting). Moreover, high loads of NMs attached onto the body caused an high

dispersion of energy due an increase of both the specific body weight and the physical resistance during swimming, which would be used otherwise to complete the moulting (Baumann et al. 2014).

Biological response, in particular oxidative stress (due to over production of ROS), caused by exposure to NMs have been demonstrated for different NMs, including CuO NMs (Pradhan et al. 2016), ZnO NMs (Ali et al. 2012), TiO_2 NMs (Sureda et al. 2018), Ag NMs (Gomes et al. 2015a), CNTs (Al-Shaeri et al. 2013). Hence, effect on cell membranes, DNA, proteins and the metabolic activity of organisms may be expected (Bundschuh et al. 2016).

In addition, toxicity of NMs can also be a result of a complex interaction between the NMs and any dispersant used during ecotoxicology studies (*e.g.* NOM and surfactants) to increase the stability of the colloidal suspensions during the experiments. These materials may adsorb to the surface of the NMs, and provide charge and steric stabilisation (Mazur et al. 2013, Adeleye et al. 2014, Amde et al. 2017), or they may hinder direct contact of the NM surface with other surfaces, solutes and organisms (Bondarenko et al. 2013).

1.1.2 Lifecycle of products containing NMs

The lifecycle of products containing NMs can be divided into four main stages: production and formulation, transport, application/use and disposal/waste/recycling (Fig. 1-3) (Miseljic and Olsen 2014, Scott-Fordsmand et al. 2017).

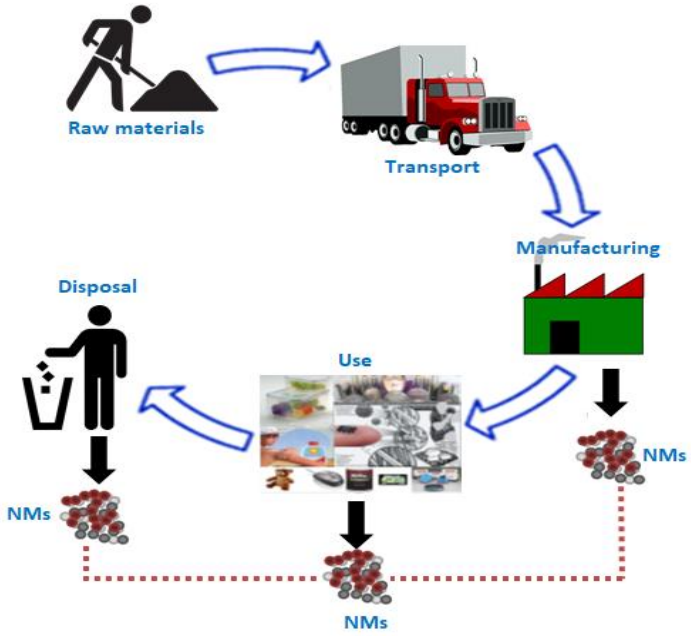

Figure 1-3 Lifecycle of NMs released in the environment from nano-enabled products from production to end-use life (based on Miseljic and Olsen (2014)).

The impact of the nano-enabled product on the environment varies during these stages depending on how the NMs are processed from raw materials and incorporated into a commercial nano-enabled product; and how this product is used, aged and/or transformed by the surrounding environment in its lifetime. To assess the potential exposure risks of a product during its entire life-cycle, a tool exists, Life-Cycle Assessment (LCA), which addresses all environmental impacts throughout its life (Miseljic and Olsen 2014).

However, when carrying out these studies on nano-enabled product, they might only be representative of human exposures occurring at manufacturing or processing sites where direct exposure to relatively pristine NMs takes place (Lowry et al. 2012). In reality, when environmental exposures occur, NMs are mostly incorporated into a matrix or present on the surface of a matrix; so, data regarding fate and effects of pristine NMs may not be particularly informative (Lowry et al. 2012, Nowack et al. 2016).

Fate and transport processes, previously described, will affect the product matrix as well as the NMs contained within it, both prior to, and after, release from the matrix. NMs will subsequently change in form and chemistry over time, so organisms will be most likely affected by the "transformed" NMs than the pristine form (Lowry et al. 2012).

The terms "weathered", "aged" and "transformed" are currently used interchangeably in the scientific literature to indicate NMs that once in the environment undergo physico-chemical transformations (Nowack et al. 2016). Nowack et al. (2012b) proposed a

classification of these materials, based on their expected environmental transformations, identifying 3 different classes:

- product-modified NMs, as NMs embedded in products;
- product-weathered NMs, as NMs transformed by environmental stressors while still associated with the product and;
- environmentally transformed NMs, as NMs released from the product and modified by environmental processes.

These 3 categories of NMs represent the most realistic form of the NMs of which humans and the environment are exposed, thus more studies investigating also their potential toxicity are needed (Nowack et al. 2012b).

The application of LCA at early stages of a nano-enabled product development can help the manufacturers in designing NMs that are safe for the environment and human health from use to disposal (Peijnenburg et al. 2015). In this perspective, recent work has been focussed on the development of "safer by design" (SbyD) NMs (Schwarz-Plaschg et al. 2017). Bottero et al. (2017) define the concept of "safer by design" as a model "*based on the integration of the life cycle assessment into managing the environmental health security (EHS) in order to optimize the benefit/risk ratio in terms of societal and economic risk*s".

The SbyD NMs production is based on the integration into the design of the safe-enabled product, the knowledge of NMs' potential adverse effects on human health and the environment, reducing exposure, minimizing waste production and safe handling and recycling (Bottero et al. 2017).

Safer by design solutions applied to NMs are essentially material surface modification strategies proposed to control exposure risk determinant properties (Costa 2016). Engineered coatings, such as polyvinylpyrrolidone (PVP), polyethylene glycol (PEG), and others are often used to prevent the natural tendency of NMs to agglomerate, due to van der Waals forces, allowing the NMs to be homogeneously dispersed when incorporated into the matrix or at the surface of the final product (Louie et al. 2016, Nowack et al. 2016). Coatings might be necessary, for instance, to enhance the product performance (*e.g*. in paints), to improve delivery of the NMs (*e.g.* subsurface remediation of environmental contaminants) or to tune reactivity of the NMs (e.g. for catalysis) (Louie et al. 2016). The agglomeration of NMs has been shown to depend on particle properties and the physicochemical properties of the media, so in the absence of surface coating (engineered or incidental) agglomeration/deagglomeration is mainly

governed by the particle core properties such as size, zeta potential and the ionic strength of the exposure medium (Miseljic and Olsen 2014).

Surface coating functionalisation can also facilitate the solubility of NMs and thus reduce their persistence in the environment and their potential toxicity (Misra et al. 2012b). Thus, the research challenge is to determine if the so modified NMs (SbyD) once released in the environment yield an increased or decreased reactivity or toxicity compared with the pristine materials (Nowack et al. 2012b).

1.1.3 Characterisation of NMs

To evaluate the relationship between concentration and response in ecotoxicological studies, it is important to describe and understand the physicochemical parameters of the tested NMs. To date, researchers have not been able to establish a single parameter that best describes this relationship, instead a variety of parameters are used in this assessment (Bouwmeester et al. 2011). As such, a comprehensive characterisation of NMs is essential in order to relate the potential toxicity of NMs in both human and environmental systems to the specific features of NMs in the complex biological media relevant to the test system (Bouwmeester et al. 2011).Thus, when performing a NM risk assessment, it is of extreme importance to characterize the NMs used in the experiments ensuring reproducibility and hence more reliability of the results (Dhawan et al. 2009).

However, extensive characterisation recommended in theory is not practically possible due to the extensive work and expensive instrumentation required. Nevertheless, the following physico-chemical proprieties are usually assessed: chemical composition, shape (although quantification might not always be possible), size, particle number and concentration (in a specific volume of medium), surface area, size distribution, surface charge/zeta potential, nature of the coatings (if present) and solubility (Amde et al. 2017).

The employed techniques used in this research project were (Ortelli et al. 2017):

- transmission electron microscopy (TEM) to detect the morphological characteristics of the NMs;
- dynamic light scattering (DLS) to determine the hydrodynamic diameter (d_{DLS}),
- electrophoretic light scattering (ELS) to perform zeta potential (ζ-potELS) measurements;
- centrifugal separation analysis (CSA) to investigate the sedimentation velocity data of the colloidal dispersions;

- inductively coupled plasma optical emission spectrometry (ICP-OES) to quantify the ions released in the medium, and
- thermogravimetric analysis (TGA) to determine the presence and amount of surface-bound ligand coverage on the NM and confirm the presence of the coatings (Mansfield et al. 2014).

1.2 Case Study: Copper and CuO NMs

Heavy metals, such as copper, are elements which can occur in nature and with an high atomic weight and a density at least five times greater than water (Tchounwou et al. 2012). Their multiple industrial, domestic, agricultural, medical, and technological applications have led to their wide distribution in the environment, raising concerns over their potential effects on human health and the environment. Their toxicity depends on several factors including the dose, route of exposure, and chemical species, as well as the age, gender, genetics, and nutritional status of exposed individuals (Tchounwou et al. 2012). Copper is also an essential trace element for all animals but the optimal range of environmental concentrations that avoids deficiency and toxicity can be rather narrow. Acute toxicity is highly variable interspecifically among species. For example within molluscs, it has been reported an LC50 ranging from 200–8000 mg L^{-1} for marine and 40–9000 mg L^{-1} for freshwater species. This difference in toxicity indicate, overall, a higher sensitivity for euryhaline species at low salinity, due to partly to differences in speciation of the metal and hence in its bioavailability and partly to interference with osmoregulation and the greater osmotic stress at low salinities (Taylor and Anstiss 1999).

Copper oxide NMs are one of the most commercially manufactured metal oxide NMs due to their optical, catalytic, mechanical, antimicrobial, and electrical properties. Due to the applications in advanced technologies, manufacturers have focused on synthesis of CuO NMs resulting in these NMs becoming one of the most extensively used inorganic materials (Singh et al. 2016). Although the production of CuO NMs constitutes a relatively small portion of the total amount of global NMs, 570 tons yr^{-1} in 2014 (predicted to be 1600 tons by the year of 2025) (Keller and Lazareva 2014, Peng et al. 2017), their widespread applications is raising concerns regarding their potential toxicity and life cycle in the environmental (Adeleye et al. 2016).

It is therefore important to study the long-term fate and transformations of NMs in aquatic systems to predict exposure and effects in environmental species.

1.2.1 Physiological aspects of copper toxicity

Copper is part of a large group of heavy metal elements (cobalt (Co), chromium (Cr), iron (Fe), magnesium (Mg), manganese (Mn), molybdenum (Mo), nickel (Ni), selenium (Se), and zinc (Zn)) that are required in only trace amounts for various biochemical and physiological functions in plants and animals (Taylor and Anstiss 1999). These elements are defined as essential metals, as they are present and maintained usually at constant concentration in all tissues. Furthermore, if inadequately supplied to the organism, they can induce a variety of deficiency diseases or syndromes (Nagajyoti et al. 2010), which, however, can be reverted if the element is reintroduced in the biological system (Simkiss and Mason 1983). The most important and well-studied functions of essential metals in the organism are: their direct participation in being an integral part of several enzymes (metallo-enzymes); their involvement on the transport of oxygen; and their important roles in various oxidation–reduction reactions (Nagajyoti et al. 2010). For example, in molluscs Zn and Mg are frequently associated with metallo-enzymes; copper-containing heamocyanin are found in free in the plasma of some gastropods (*e.g. Lymnaea stagnalis*) (Dawson et al. 1981); and Fe-/Cu-containing cytochrome systems (redox and electron-transfer systems) are incorporated into the mitochondrial system of lamellibranches and gastropods (Simkiss and Mason 1983).

In contrast, non-essential trace elements, such as mercury (Hg), silver (Ag), lead (Pb) and nickel (Ni), are heavy metals that are highly toxic and they neither break down in the environment nor easily metabolized determining bioaccumulation in the organisms and biomagnification on the trophic chain (Tchounwou et al. 2012).

Heavy metals are also called trace elements due to their presence in trace (10 $mgkg^{-1}$, or mgL^{-1}) or in ultra-trace (1 μgkg^{-1}, or μgL^{-1}) quantities in the environmental matrices (Nagajyoti et al. 2010). Some of these elements, such as iron, although ubiquitously present in the environment, are scarcely bioavailable to organisms due to their low solubility. Their cellular uptake occur through different mechanisms, such as induction of the synthesis of specific proteins (*e.g.,* metallothioneins), or passage through the endoplasmic reticulum, mitochondria, Golgi systems, and lysosomes (Taylor and Anstiss 1999). For example, molluscs can selectively taking up or rejecting particular metals through their high permeable epidermis, wherever it is not protected by a shell, and at the digestive and respiratory epithelia.

Nevertheless, numerous species have evolved storage systems to ensure a consistent present of these elements in their systems, avoiding cellular deficiencies. For example, molluscs form intracellular granules which can equilibrate the amount of metals in the

blood (*e.g.* digestive gland granules in *L. stagnalis* (Desouky 2006)), or extracellular granules which containing a wide range of metal ions (*e.g.* rhogocytes in *Helix pomatia* (Dallinger et al. 2005)).

However, due to the high reactivity of heavy metal ions, if in excess, can cause cellular and tissue damage in different cell components (e.g. cell membrane, mitochondrial, lysosome, endoplasmic reticulum, nuclei), leading to a variety of adverse effects and diseases, such as cell-cycle modulation, carcinogenesis, or apoptosis (Simkiss and Mason 1983).

A good example of these effects is provided by copper. In molluscs, trace amounts of copper are essential to form the metallo-proteins, but its inhibitory and toxic effects are well known from its use as molluscicides on terrestrial and aquatic species (Simkiss and Mason 1983). An excess of copper bound to macromolecules can perturb their biological function directly or it may displace other metals such as zinc from apoenzymes (Taylor and Anstiss 1999). However, the most critical aspect of the toxicity of Cu ions is their capacity to cycle between Cu(II) and Cu(I), which can result in the generation of superoxide and hydroxyl radicals (reactive oxygen species (ROS)) from intracellularly generated hydrogen peroxide, via Fenton chemistry, catalysing the Haber–Weiss reaction, which normally is extremely slow (Kehrer 2000), following this reaction:

Cu(II) (M^{n+}) + $^{\cdot}O_2^-$ → Cu(I) (M^{n+-1}) +O_2 (1)

Cu(I) (M^{n+-1}) + H_2O_2→ Cu(II) (M^{n+}) + OH^-+ $OH^{\cdot}$ (Fenton reaction) (2) (M = metal)

The net reaction: $^{\cdot}O_2^-$ + H_2O_2→ O_2+ OH^-+ $OH^{\cdot}$ (Haber–Weiss reaction) (Goldstein and Czapski 1986).

As a result, ROS, if in excess, might provoke oxidative stress which is defined as "*a disruption of the prooxidant–antioxidant balance in favour of the former, leading to potential damage*" (Kehrer, 2000). Cells possess a variety of antioxidant defences that could protect against copper-induced (or in general metal-induced) oxidative damage, such as the enzymes superoxide dismutase (Cu-SOD), catalase and glutathione peroxidase (GPx), which limit the formation of ROS. Removal of ROS from the cytosol can also occur via non-enzymatic antioxidants such as ascorbate (ASC) and glutathione (GSH); or by copper chelators such as metallothioneins (Taylor and Anstiss 1999).

One of the major uptake source of waterborne copper for many aquatic organisms is the respiratory organ. Exposure to toxic levels of copper can cause gills hypertrophy and their epithelium necrosis (*e.g.* Al-Bairuty et al. 2013), and a reduction in the activity of the Na/K ATPase ion transporter (Griffith 2017) . Ion-regulation imbalance due to

copper exposure has been also demonstrated in aquatic gastropod exposed to copper. Brix et al. (2011) exposing juveniles of *L. stagnalis* to Cu ions ($CuSO_4$), attributed the toxicity of Cu to the inhibition Ca^{2+} and Na^{2+} uptake, which being essential and highly required for snails at this development stage (Ebanks and Grosell 2008). Uptake from food and sediments are also important route of exposure and entry of copper depend on the feeding strategy and level of metal contamination of the water (*e.g.* Pang et al. 2012, Croteau et al. 2014b). Continually uptake and low excretion rates of copper have been demonstrate to cause, as well, bioaccumulation of the metal for many marine and freshwater species, of which rate is usually species and environment specific (Simkiss and Mason 1983). In molluscs, copper taken up from the environment is accumulated and compartmentalized mainly in epithelial cells of the midgut glands of gastropods and cephalopods and in the kidney and gills of bivalves, reducing so the toxic effects of the metal. For example, Ng et al. (2011) during chronic exposure to Cu ions, in the presence of food, found no significant mortality but an inhibition of growth of juveniles of *L. stagnalis*; due to Cu^{2+} detoxification mechanisms evidenced by increases in metallothionein-like protein concentrations and Cu^{2+} binding to metal-rich granules in the soft tissues (Ng et al. 2011). Finally, in many crustaceans and molluscs, circulating heamocyanin is one of the largest copper-binding pool, which, at relative constancy of haemolymph volume, can assist in the variation of copper in the different cell compartments (Marigómez et al. 2002).

1.2.2 Ecotoxicity of CuO NMs

Although CuO NMs are one of the most commercially used metal oxide NMs and, at present, are not included in the OECD NMs' list (OECD 2010), due to the rising interests in this material from industry, environmental releases are likely to increase. Therefore, it is important that further research on these materials take place.

Environmental research into CuO NMs' toxicity has mostly focused on the effects on organisms, especially those in aqueous environments. Ivask et al. (2014), using an approach that adheres to the EU-Directive 93/67/EEC (EU 1993), which underlines the principles for assessment of risks to humans and the environment of chemical substances, reviewed the toxicity of pristine CuO NMs on a test battery of crustaceans, algae and fish. These authors classified CuO NMs as 'toxic' to aquatic organisms, L(E)C50 values were estimated to be around 2000–3000 μgL^{-1} CuO, according to the lowest median L(E)C50 value for the three key aquatic test organisms. Interestingly, these authors found that when pristine CuO NMs were used as biocidal agent, non-

target organisms were more affected than the target ones. Consequently, if the use of CuO NMs continues to increase, their discharge and subsequent leaching to surface waters may pose increasing threats to aquatic species (Ivask et al. 2014).

It is still disputable if the soluble Cu or the nano mediated effects are the most important factor in the toxicity of CuO NMs. Indeed, most of the research related to CuO NMs toxicity to organisms crucial in aquatic food webs like microalgae (Perreault et al. 2012, Melegari et al. 2013), protozoa (Blinova et al. 2010, Ivask et al. 2014), bacteria (Moore et al. 2017) and invertebrates (Adam et al. 2015, Pradhan et al. 2012), attributed their toxicity to the leached ionic form of the metal NMs.

For example, Torres-Duarte et al. (2016) showed that after 96h waterborne exposure, medium and internalized dissolution of two different CuO NMs (a lab pure synthesized and a less-pure commercial form) caused detrimental effects on the morphology, development and survival of the white sea urchin, *Lytechinus pictus* as a result in changes to the redox environment (Torres-Duarte et al. 2016). Similar conclusions were drawn by Adam et al. (2015) studying the chronic toxicity of CuO NMs to the freshwater crustacean, *Daphnia magna*. Chronic adverse effects on the reproduction and growth (in length) of adults were attributed to copper ions formed during dissolution of the NMs in the exposure medium (Adam et al. 2015).

However, in contrast, other research studies demonstrated that the toxicity might be attributed to a NMs-specific effect (Amorim and Scott-Fordsmand 2012, Croteau et al. 2014b) or to a combination of ions and NMs toxicity (Mwaanga et al. 2014, Gomes et al. 2015b).

Amorim and Scott-Fordsmand (2012) compared the toxicity of Cu NMs and ionic Cu (as $CuCl_2$) on survival, reproductive output and avoidance behaviour of the enchytraeid, *Enchytraeus albidus.* A higher reprotoxicity and avoidance behaviour, for the worms exposed to Cu NMs compared to those exposed to the ionic form of Cu, were attributed to a NM-specific effect since only a small fraction of ions was released from the NMs (Amorim and Scott-Fordsmand 2012). Furthermore, two studies investigating the effects of CuO NMs to the freshwater pond snail, *Lymnaea stagnalis* (Misra et al. 2012a, Croteau et al. 2014b), indicated that dissolution had a negligible influence on the bioaccumulated Cu. These authors used stable isotope tracing techniques to determine Cu uptake. Both studies investigated the bioaccumulated Cu from CuO NMs, demonstrating that use of non-labelled NMs will be very difficult to allow clear interpretation of results due to the high level of Cu background in the tissues of this species. Misra et al. (2012a) exposed *L. stagnalis* to concentrations equivalent to the

background of Cu concentrations in freshwater systems (0.2−30 μgL^{-1}), in order to gain a mechanistic understanding of CuO NMs and their uptake and toxicity at environmentally and physiologically realistic concentrations. The net uptake of ^{65}Cu in *L. stagnalis* was found to be linear over a wide range of concentrations that encompasses what are considered realistic environmental exposures (i.e. <150 ppb). Croteau et al. (2014b) investigated the uptake of ^{65}Cu by *L. stagnalis* after aqueous and dietary exposures to ^{65}CuO NMs. They estimated that, in their experimental conditions, 80-90% of the bioaccumulated Cu concentration originated predominantly from the ^{65}CuO NMs. The physiological loss of ^{65}Cu incorporated into tissues after exposures to ^{65}CuO NMs, over 16 days of depuration time, was of 44% of the accumulated ^{65}Cu over the first 4 days of depuration and not detectable thereafter. As a result, large Cu body concentrations are expected in *L. stagnalis* after exposure to CuO NMs.

In contrast, Mwaanga et al. (2014) exposing *D. magna*, to sublethal concentrations (300, 800 and 1100 μgL^{-1}) of CuO NMs found that the observed oxidative stress was induced by both metal ions released from the metal oxide NMs and NMs themselves (Mwaanga et al. 2014).

In general, studies show a lower toxicity of CuO NMs in freshwater rather than in seawater, with authors attributing this difference to the slower dissolution at low ionic strengths and Cu^{2+} complexation in the presence of organic matter (Adeleye et al. 2014). Ca^{2+} and Mg^{2+} cations (that cause water hardness) are known to mitigate the toxicity of metal ions by competing with them for the binding sites on biological membranes (Brix et al. 2011).

As previously stated, CuO NMs toxic effects are dependent on the different physico-chemical process which the NMs can undergo such as dissolution, aggregation/agglomeration and precipitation, which are highly dependent on particle size, surface characteristics, exposure environment and exposure routes (Torres-Duarte et al. 2016).

Rippner et al. (2018) showed that although the presence of DOM in the exposure medium promoted dissolution of CuO NMs, it elicited also a reduction of growth inhibition for a model aquatic plant duckweed, *Landoltia punctate*, compared to the growth inhibition induced by the ionic form of Cu ($CuCl_2$). Indeed, the solubility of CuO NMs depends on their interactions with organic material in the exposure environment (*e.g.* proteins, amino acids, natural organic matter, and humic substances) that may coat and disperse NMs or complex metal ions. Furthermore, these authors studied also the effects of size (22 nm and 50 nm) of the NMs on the dissolution and

toxicity of CuO NMs. Higher dissolution of 22 nm CuO was observed compared to 50 nm CuO which led to greater Cu bioavailability and uptake, inhibiting duckweed growth to a greater extent (Rippner et al. 2018). Particle size dependent toxicity was previously demonstrated for CuO NMs by Heinlaan et al. (2008), which attributed a size-related higher toxicity of CuO NMs compared with the bulk counterpart to *D. magna*. LC50 values ($LC50_{48h}$ = 3.2 mgL^{-1} Cu) estimated for CuO NMs were about 50 times fold lower than for its bulk form.

As previously described, studies have shown that CuO NMs tend to agglomerate and deposit onto the sediment in the aquatic ecosystem (Buffet et al. 2011, Buffet et al. 2013) potentially posing a high biological risk for benthic organisms. Further chemical, physical and biological mechanisms (like bacterial decomposition or bioturbation) occur at the sediment level (Hanna et al. 2013) transforming these NMs and influencing their potential toxicity. Indeed, Hanna et al. (2014) examining the long-term (30 days) accumulation and toxicity of CuO NMs (1–3 mgL^{-1}) to the marine mussel, *Mytilus galloprovincialis,* found that this organism could biotransform the CuO NMs. At the higher concentration (3 mgL^{-1}) mussels rejected and excreted CuO NMs in bio-deposits containing approximately 27000 times the CuO NMs exposure concentration, reaching as much as 110 mg Cu g^{-1}, suggesting the potential for biomagnification in benthic organisms (Hanna et al. 2014).

As indicated above, organisms are most likely exposed to the "transformed" NMs instead of the pristine form. Hence, the toxicity that these NMs may display will require careful evaluation and testing (Kumar et al. 2012). Moore et al. (2016) evaluated the effects of pristine and transformed Cu NMs on superficial sediment microbial communities in simulated freshwater wetlands. Mesocosms were dosed through the water column with a high concentration (2.5 gL^{-1}) of CuO (31.1 ± 12 nm), CuS NMs (12.4 ± 4.1 nm) or Cu ions and impacts on the microbial communities were examined over 10 months. After 30 days of exposure, NM-dosed mesocosms had relatively stable microbial community's densities. In contrast, the mesocosm spiked with Cu ions revealed a decrease of nearly 5-time fold at day 90 and a decrease by 2 orders of magnitude at day 180 of the microbial biomass. However, by 300 d, bacterial community cell densities of all the mesocosms had recovered, converging with one another, including the control mesocosm. This study suggested that within chemically and biologically complex systems environmental factors are likely to mitigate the long-term effects of these NMs on microbial communities (Moore et al. 2016).

Nevertheless, to ensure the safe use of CuO NMs, efforts have been made to reduce the toxicity of CuO NMs by using a SbyD approach controlling their physico-chemical proprieties (*e.g.* particles' diameter, agglomeration behaviour, modifying surface characteristic) (Hou et al. 2017a).

In the specific case of CuO NMs, very few studies that have investigated the effectiveness of SbyD strategies on of their toxicity, with results showing contrasting and wide-ranging findings. Indeed, evaluating the microbial activity of PVP-coated and non-coated CuO NMs, Javed et al. (2017) demonstrated that the coated CuO had significantly better activity against bacterial strains than uncoated CuO. In contrast, Clar et al. (2016) studying the toxicity on Cu-PVP on microbial communities utilized in biological wastewater treatment (BWT) system, found that the presence of a PVP coating on Cu NMs had little effect on the microbial inhibition measured, attributed mainly to the release of ionic Cu in solution via catalytic reaction promoted by reduced Cu^{+} species.

In contrast, Naatz et al. (2017) showed the efficacy of a SbyD limiting the dissolution of CuO NMs by doping them with different concentrations of Fe (1–10%) in a flame spray pyrolysis reactor. Toxicity tests performed in tissue culture cell lines and zebrafish embryos demonstrated that with increased levels of doping there was a progressive decrease in cell-line cytotoxicity, as well as an incremental decrease in the rate of hatching interference in zebrafish embryos.

In contrast, Perreault et al. (2012) found that the use of a polymer coating on core–shell (CS) CuO NMs to decrease NM aggregation resulted on a higher toxicity to the green alga *Chlamydomonas reinhardtii* compared to bare CuO NMs after 6 h treatment at the same concentrations (5–40 mgL^{-1}). The higher toxicity of CS-CuO NMs was shown to be mainly caused by their higher NMs uptake, which accumulated as large aggregates inside membrane structures in the cytosol, due to the smaller size of CS-CuO NMs (HDD= 65.4 nm) compared to the larger bare CuO NMs (HDD=148nm) aggregates (Perreault et al. 2012). Thus, the research challenge is to determine if the modified NMs once released in the environment yield an increased or decreased reactivity or toxicity compared with the pristine materials (Nowack et al. 2012b).

This research project approach was to perform a series of acute and long term ecotoxicological tests to investigate the toxicity of CuO NMs at various stages of their life cycle (pristine, fragmented product or after modification for the purpose of SbyD). These experiments were supported by a systematic characterisation of key relevant

properties of the NMs, to identify links with physicochemical surface modifications (Costa 2016).

In this research project, experiments were focussed on acute and chronic toxicity of the CuO NMs on the benthic ecosystem, using the pond snail *L. stagnalis* as representative species. Acute and chronic exposure experiments were performed using different approaches and biological endpoints, such as mortality, reproduction and behaviour studies, and gene expression of antioxidant enzymes under environment stressors. Ecotoxicology experiments were performed using pristine CuO NMs, modified CuO NMs with Na ascorbate (ASC) and Polyvinylpyrrolidone (PVP) and finally CuO Fragmented Product (FP), where the NMs were incorporate in a matrix and then fragmented.

1.2.3 Fate and behaviour of CuO NMs in the aquatic environment

At present, CuO NMs' environmental risk is considered to be low, given that average exposure concentrations are predicted to be in the μgL^{-1} range (Keller et al. 2017). Although CuO NMs can enter the environment via several routes, the majority of the releases are attributed to their use as biocidal agents in marine antifouling paints or agricultural biocides, which are directly introduced into the environment as intentionally toxic substances (Conway et al. 2015). In addition, CuO NMs may occur as well as an "incidental" particle generated by chemo-mechanical polishing operations on copper-containing substrates in the semiconductor industry (Wang et al. 2013).

Keller et al. (2017) estimated, based on the global market, that up to 0.25 million tons yr^{-1} of Cu may be released in the environment due to the use of Cu NMs as biocidal agent. This could increase dramatically the potential accumulation of Cu in localized hot spots, where they may eventually reach and surpass the lowest observable effect concentrations (LOECs) (Keller et al. 2017, Caballero-Guzman and Nowack 2017) (Fig. 1-4).

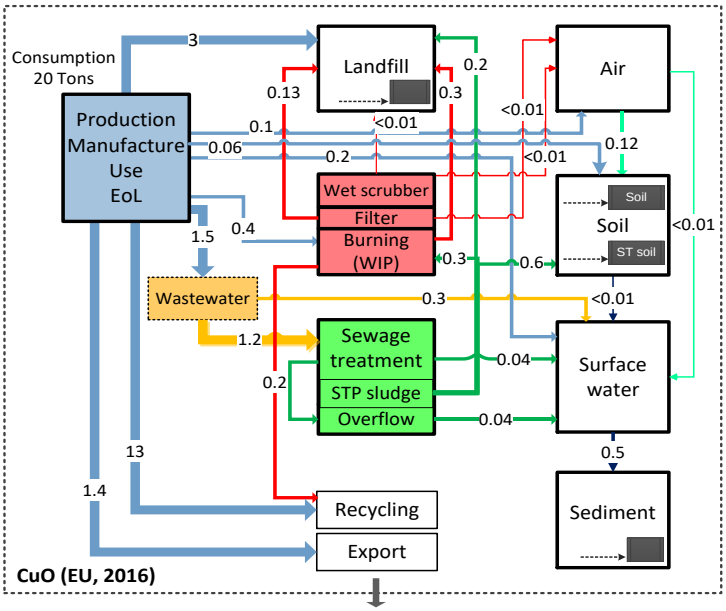

Figure 1-4 Total flows (all life cycle stages) of CuO NMs used in all nano-enabled product categories. The values correspond to the means of the probability distributions simulated. Units: tonnes per year (Caballero-Guzman and Nowack 2017).

Release of CuO NMs from painted surfaces is ruled mainly by NMs' chemistry and environmental factors, nevertheless the surface structure on which the paints are applied might also lead to additional leaching (*e.g.* wood). Caballero and Nowack (2017) (unpublished work within the SUN consortium) estimated, for the wood fence coated with CuO NMs during manufacturing and use stages, a concentration release in sediments around 0.2 µg kg·yr^{-1}.

Platten et al. (2016) examined the release of copper and copper micro/NPs from lumber pressure-treated with micronized copper, using a surface wipe method to simulate dermal transfer and potential ingestion through hand-to-mouth, over one year outdoor weathering period. The amount of copper released was high in the first two months, but decreased to a constant level (~1.5 mgm^{-2}) afterwards. The authors estimated a release, via dermal contact, of 2.58 mg of copper which exceeds the tolerable upper intake limit for children under the age of 8, based on the total dietary copper intake (Platten et al. 2016).

These results highlight the need for a detailed understanding of potential material release from nano-enabled products during realistic exposure scenarios. Indeed, in the environment, the transformation of CuO NMs is controlled by the chemistry of both the particles and the environment. For example, CuO NMs released from consumer products, passing through waste water treatment plants may be further transformed/complexed by organic matter into less toxic forms of Cu (Keller et al. 2017).

Adeleye et al. (2014) investigated the effects of NOM such as extracellular polymeric substances (polysaccharides, proteins, nucleic acids, and other polymers) (EPS) on the stability and dissolution of copper NMs. They found that different Cu NMs (*e.g.* Cu NMs, CuO NMs, kocide) show a higher dissolution rate at low acidic pH 4. Over 90 days of exposure, CuO NMs showed the highest dissolution rate of 21%, only after 2h in distilled water. In contrast, in the presence of NOM, CuO NMs exhibited the lowest dissolution rate, reaching the 98% dissolution rate after around 21−30 days of exposure. In fact, dissolution of CuO NMs has been shown to increase with the increases in ionic strength (IS) of the medium (Adeleye et al. 2014).

Cu ions are recognized to be toxic to many organisms, thus a lower dissolution of the NMs may reduce their overall hazard. Indeed, dissolved Cu^{+} released, in the environment, by some Cu NMs are readily oxidized to Cu^{2+} and then bound by other ions or molecules forming inorganic complexes such as sulphate, sulphide, phosphate, chloride, and carbonate (Adeleye et al. 2016).

Conway et al. (2015) quantified physiochemical behaviours of three different species of Cu-based NMs (Cu NMs, CuO NMs and $Cu(OH)_2$) in eight natural and artificial waters covering a range of IS, pH, and organic content. The fate of the three NMs was governed by different water properties, but the presence of phosphate appeared to be the main controlling factor for nano-CuO sedimentation. This was due to the shift of surface charge (from positive to negative) by covalent binding the CuO, and thus influencing the fate and toxicology of these NMs by altering their interactions with ions and other charged surfaces (Conway et al. 2015).

These studies were performed under almost saturated conditions, where the maximum solubility of CuO NMs may be reached. In unsaturated conditions, Kent and Vikesland (2016) demonstrated that in respect to Cu^{2+}, CuO NMs are not environmentally persistent, even in freshwater at pH 7.7; where, instead, the results suggest that Cu_{ox} NMs lifetime could range from several minutes to a couple of hours. However, in this study experiments were performed using an atomic force microscope (AFM) where a continuously flowing solution flushed dissolved species from the AFM fluid cell so that under-saturated conditions were constantly maintained. Thus, with this setup it was not possible to evaluate the persistence of the Cu NMs agglomerates, which normally form in a realistic environment, since agglomeration would most likely not take place (Kent and Vikesland 2016).

A study by Vencalek et al. (2016) went a step further and evaluated the dissolution rates of two copper-based NMs (CuO or $Cu(OH)_2$ (pesticide Kocide 3000; 1000 μgL^{-1}) in

natural (freshwater wetland mesocosm, pH 7.7) and artificial waters (deionized water, pH 5.8) under both saturated and unsaturated conditions. Overall, $Cu(OH)_2$ NMs dissolved more rapidly than CuO NMs. The effective dissolution rate constant and experimental half-life of the NMs were calculated from model fits of the dissolution data, estimating an experimental half-life of <8 h in natural water for $Cu(OH)_2$ NMs and of 73 h in natural water for CuO NMs. The dissolution of both NMs proceeded at a slightly faster rate at lower pH (pH 5.8) in under saturated ligand-free deionized water. At higher pH (pH 7.7) values, in oversaturated natural waters, the dissolution proceeded coupled with the increase of DOC concentration (Vencalek et al. 2016). Although these data suggest that in a real environment copper based NMs are unlikely to become persistent pollutants in their nanoscale form, it is important to note that no agglomeration phenomena were taken into consideration in this study.

Peng et al. (2017) studied the agglomeration, sedimentation, dissolution, and speciation of CuO NMs by varying environmental parameters including pH, IS, ionic valence (monovalent (NaCl), and divalent ($CaCl_2$)), and NOM. Findings showed that in laboratory conditions the pH of the exposure environment determined the surface charge of CuO NMs. The increase in IS and the ionic valence of electrolyte in solution, promoted the agglomeration and sedimentation of the CuO NMs. Furthermore, the presence of $CaCl_2$ slowed down the dissolution rates of the materials compared to the conditions in the presence of the monovalent electrolyte (NaCl). Finally, in agreement with other authors (*e.g.* Adeleye et al. 2014), NOM reduced the sedimentation rate of the CuO NMs in a dose-dependent manner (Peng et al. 2017).

In a similar study, Xiao et al. (2018) studied the associated variation of the same environmental parameters (pH, IS, ionic valence and NOM) on the fate and behaviour of Cu NMs to assess what might happen in real natural environments where the change in multi-environmental parameters may occur simultaneously. Results confirmed that the addition of divalent cations was the most influential factor in the aggregation of Cu NMs due to the net attractive force between particles as a result of prevailing Van der Waals attraction, followed by the dissolved organic carbon (DOC) concentration which explained around 23% of the variation in aggregate size of the Cu NMs. The interaction between the divalent cations and DOC was also important in influencing the aggregation of Cu NMs, due to modification of the NMs' surface charge and the formation of aggregates attributed to the bridging effects between divalent cations and DOC. Dissolution was predominantly influenced by the DOC concentration, which was far more important than the effect of pH in the 6-9 on the dissolution of Cu NMs. In

addition, the sedimentation profile of Cu was more influenced by the aggregation behaviour of Cu NMs than by their dissolution (Xiao et al. 2018).

In conclusion, CuO NMs once in the environment can undergo degradation and transformation mediated by organisms' activities. For example, Kovačec et al. (2017) investigating the comparative toxicity of ionic Cu, micro CuO and CuO NMs used as fungicides on the plant pathogenic fungi, *Botrytis cinerea*, found that the organism was able to biotransform the nano and micro CuO, leading to a reduction in toxicity. *B. cinerea* induced leaching and mobilization of Cu ions from the particles and their further complexation with extracellularly secreted oxalic acid forming a blue compound identified as Cu-oxalate complex (Kovačec et al. 2017).

1.2.4 Characterisation of CuO NMs tested

Characterisation of the physico chemical proprieties of the NMs tested in this research project was not performed by the author of this thesis. Instead, SUN project Partners, the University of Venice, and ISTEC-CNR (Faenza, Italy), analysed and characterised the NMs in different environmental media.

Characterisation of the CuO NMs tested was performed both in Milli-Q water and in the medium used for the experiments, namely the OECD 203 medium (OECD 1992), at different time points and under the same experimental conditions used during the ecotoxicological studies. The data reported by the two SUN partners are summarised in this section, with interpretation and discussion provided by the author of this thesis.

1.2.4.1 Pristine CuO NMs

Pristine copper oxide NMs (CuO NMs) (CAS-Number: 1317-38-0) produced by PlasmaChem GmbH© were provided either in the powder or dispersed form. To measure most of the proprieties of the NMs using the instrumentation previously described, samples needed to be in liquid suspension or dispersion.

At the University of Venice, probe sonication was used to prepare a stable dispersion solution (SUN 2014a). At ISTEC-CNR, a different method was used to prepare the stock suspension of pristine CuO NMs (SUN 2015) in order to promote stabilisation; the suspension with CuO NMs was left for 95 hours stirring with the aid of 3 mm zirconia spheres (grinding bodies). Morphological characterisation of NMs was performed by Transmission Electron Microscopy (TEM) (FEI Tecnai 12 G2) (see Appendix A, subsection A.1 for more details).

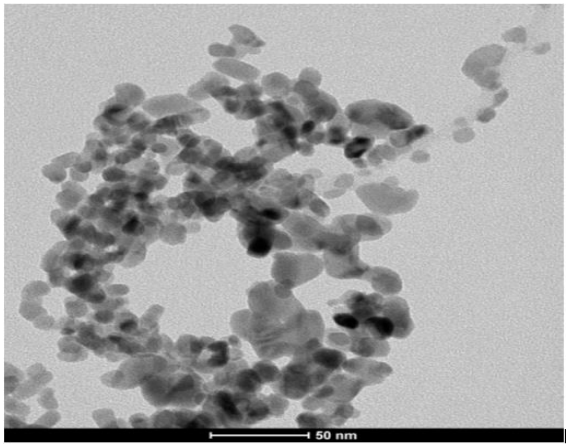

Figure 1-5 Representative TEM micrographs of pristine SUN CuO NMs (av. magnification 310,000) (SUN 2014a).

Results summarized in Tab. 1-1 and showed in Fig. 1-5 showed an estimated average size of 12 nm in diameter when suspended in Milli-Q water.

Table 1-1 Observation and measurement results of TEM primary size distribution of pristine CuO NMs (SUN 2014a).

Material	**Av. Magnification (min-max)**	**Particle size distribution**		
		method	*Mode (1^{st}-3^{rd} quartile) (nm)*	*Primary size distribution Min---Max (average) [nm]*
Pristine CuO NMs	310,000 (235,000-650,000)	Manual diameter	10 (9.2-1.4)	3-35 (12)

The hydrodynamic size (d_{DLS}) and the surface charge (ζ-pot_{ELS}) of the NM dispersions were characterised with a ZetaSizer Nano ZS (Malvern Instruments Inc., UK) utilizing dynamic light scattering (DLS) and electrophoretic light scattering (ELS), respectively. At the University of Venice, z-average and z-potential of pristine CuO NMs in Milli-Q water were measured. Data show a z-average of 135.3 nm with a polydispersity index of 0.0224, denoting a monodisperse distribution (Tab. 1-2) (SUN 2014a).

Table 1-2 Water medium dispersibility results (SUN 2014a).

Material	**D_{50} by intensity (nm)**	**Mean intensity (nm)**	**Z-average (nm)**	**Polydispersity index**
Pristine CuO NMs	139.5±4.6	152.4±7.5	135.3±3.8	0.0224±0.023

Z-potential was determined either in ultrapure water or in a buffered electrolyte solution at pH 7 (SUN 2014a). The results of z-potential in ultrapure (UP) water and at pH 7 of pristine CuO NMs are summarised in Tab. 1-3.

Table 1-3 z-potential results of pristine CuO NMs (SUN 2014a).

Medium	**pH after measurements**	**Z-potential (mV)**	**Mobility (µm/sec/v/cm)**	**Conductivity(mS/cm)**
Ultrapure water	6.84	+28.1 ± 0,6	2.203 ± 0.04	0.008 ± 0.01

Buffered water at pH7	7.01	-19.2 ± 1.3	1.296 ± 0.09	31.2 ± 1.5

Sample dispersions in UP water showed z-potential close to the stability value of ±30mV (+28mV); the presence of the buffered solution instead determined a change in charge of the zeta potential, becoming negative, highlighting that the electrolyte in solution promote agglomeration resulting in a lower absolute value of zeta potential of -19mV. Furthermore, all samples show relatively low electrophoretic mobility, leading to large noise, which required a high number of sub-runs for measurement.

Sedimentation velocity data of the colloidal dispersions using the multi-wavelength dispersion analyser LUMiSizer® 651 (LUM GmbH) could not be estimated due to the very low dispersion stability (see Appendix A, subsection A.2 for more details).

At ISTEC-CNR the hydrodynamic diameter, particle size distributions (PSD) by intensity (via dynamic light scattering, DLS) and z-potential (via electrophoretic light scattering, ELS) of the working stock suspension diluted in OECD 203 medium were assessed by using a ZetaSizer nano ZSP (model ZEN5600, Malvern Instruments, UK). Both particle size distribution and zeta potential analyses were performed in triplicate (Tab. 1-4) (SUN 2015).

Table 1-4 Size z-average (d_{DLS}) and z potential data of pristine CuO NMs sample dispersed in OECD 203 medium (SUN 2015).

Material	d_{DLS} (nm)	Zeta-pot $_{ELS}$ (mV)
Pristine CuO NMs	2558 ± 472	-4.36 ± 0.57

Despite the fact that the stock suspension was prepared in Milli-Q water, a negative zeta potential was obtained. It suggested that the salts present in OECD 203 medium lead to a specific adsorption of anions onto the CuO NMs surface. Only in the case of CuO NMs stock suspension prepared and dispersed in Milli-Q water, it is possible obtain a positive zeta potential, as shown in the table above (Tab. 1-4).

Furthermore, at ISTEC-CNR, ICP-OES analysis was carried out to calculate the $Cu_{dissolved}/CuO_{total}$ ratio (%) of pristine CuO NMs, which is useful to evaluate the dissolution in the OECD medium 203 during the experiments. Findings indicate a poor dissolution in OECD 203 medium of the pristine CuO NMs analysed at the concentration of 50 mgL^{-1} Cu (Tab. 1-5) (see Appendix A, subsection A.4 for more details).

Table 1-5 $Cu_{dissolved}/CuO_{total}$ % of sample dispersed in OECD 203.

	$Cu_{dissolved}/CuO_{total}$ (%) OECD 203 (50 mgL^{-1})	
Pristine CuO NMs	t = 1h	t = 24h

	0.121	< 0.02

1.2.4.2 Safe by design CuO NMs

The SUN partner, ISTEC-CNR, designed different CuO NMs modified with various coatings in order to examine their ability to act as SbyD NMs, with the assumption that through manipulation of physical and chemical properties of pristine engineered NMs, potentially less toxic materials could be manufactured, maintaining their valuable functional properties (Costa 2016).

Five different NMs were provided varying in the dispersion media (Milli-Q water and phosphate buffer saline (PBS)) with two different surface coating agents, polyvinylpyrrolidone (PVP- M_w 29000, Sigma-Aldrich) and sodium ascorbate (ASC- M_w 198.11, Sigma-Aldrich) (Fig. 1-6).

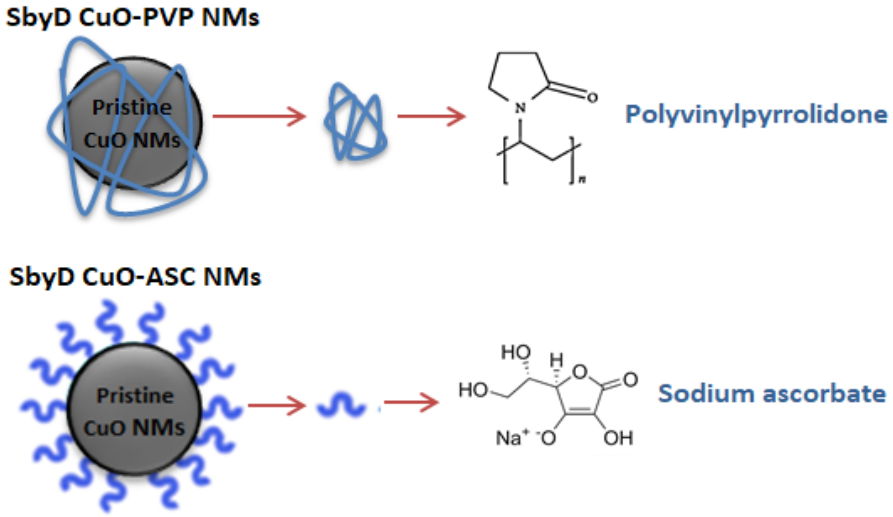

Figure 1-6 Schematic representation of the SbyD CuO NMs functionalised by self-assembling using two different modifying coatings, ASC and PVP. Figure adapted from Ortelli et al. (2017).

The PBS solution was used as a dispersant due to the well-known ability of phosphate to covalently bond to metal oxide giving the potential to significantly alter their surface properties (Conway et al. 2015).

Colloidal properties and dissolution behaviour were assessed by preparing a stock dispersion at 10 gL^{-1} Cu (1 wt %), in either Milli-Q water or PBS and diluting at a working stock concentration in OECD 203 medium. The following suspensions were produced:

- $CuO(PO_4)^{3-}$ NMs (pristine CuO in PBS);
- $CuO\text{-}PVP(PO_4)^{3-}$ NMs (SbyD (PVP) CuO in PBS);
- $CuO\text{-}ASC(PO_4)^{3-}$ NMs (SbyD (ASC) CuO in PBS);

- CuO-PVP(H_2O) NMs (SbyD (PVP) CuO in Milli-Q water);
- CuO-ASC(H_2O) NMs (SbyD (ASC) CuO in Milli-Q water).

Hydrodynamic diameter by intensity (via DLS) and z-potential (via ELS) of the working stock suspension diluted in OECD medium were measured with a ZetaSizer nano ZSP (model ZEN5600, Malvern Instruments, UK). Data from pristine and modified CuO samples diluted in OECD 203 were compared with those obtained prior to dilution; results are reported in Tab. 1-6 and 1-7.

Table 1-6 Size data (zeta-average) of CuO NMs samples diluted in Milli-Q water and OECD 203. *Data acquired from (Ortelli et al. 2017).

	d_{DLS} (nm)		
	Milli-Q water	**OECD 203 in PBS**	**OECD 203 in Milli-Q water**
Pristine CuO NMs	1093 ± 50	2364 ± 282	2558 ± 472
SbyD (PVP) CuO	797 ± 84	2098 ± 545	1159 ± 256*
SbyD (ASC) CuO	122 ± 1.4	1719 ± 157	1293 ± 278*

The data in Milli-Q confirmed the expected effect of the surface modifiers addition. In fact, samples coated with ionic agents (negative ASC) were better dispersed with a significant reduction in average hydrodynamic diameter size in comparison with the pristine sample. Neutral PVP did not improve significantly the dispersion of pristine CuO NMs.

Data collected from samples diluted in OECD 203 confirmed the presence of agglomeration, justified by the increase of ionic forces due to the salts content in OECD 203, particularly noticeable in negative ASC modified samples that stabilize CuO NMs water dispersion by improving the repulsive potential due to the negative potential transferred to particle surface. The samples previously not well dispersed (pristine and PVP modified), instead, showed a further worsening of dispersion.

Table 1-7 Zeta potential of CuO NMs samples diluted in Milli-Q water and OECD 203 medium. *Data acquired from (Ortelli et al. 2017).

	ζ-pot_{ELS} (mV)		
	Milli-Q water (pH=6.5)	**OECD 203 in PBS** (pH=7)	**OECD 203 in Milli-Q** water (pH=8.1)
Pristine CuO NMs	-9.1 ± 0.4	-3.37 ± 0.1	-4.36 ± 0.57
SbyD (PVP) CuO	-8.1 ± 2.3	-6.68 ± 0.1	+1.6 ± 0.3*
SbyD (ASC) CuO	-17.4 ± 0.3	-9.52 ± 0.2	−8.1 ± 0.4*

Overall, the dispersion in OECD 203 did not modify the charge, except for CuO PVP NMs in Milli-Q. In the latter sample, a slight increase was observed due to the stabilisation driven by the polymers. In general, decrease in colloidal stability of data in OECD 203 was expected by the improved ionic strength of the medium, confirming the PSD measurements.

At the University of Venice, sedimentation velocity data of the colloidal dispersions were obtained from centrifugal separation analysis (CSA), using the multi-wavelength dispersion analyser LUMiSizer 651® (Ortelli et al. 2017) (see Appendix A, subsection A.3 for more details). Data were obtained only for SbyD CuO NMs dispersed in Milli-Q water and diluted in OECD medium. Data were reported in Ortelli et al. (2017), and are here briefly summarised and presented in Fig. 1-7.

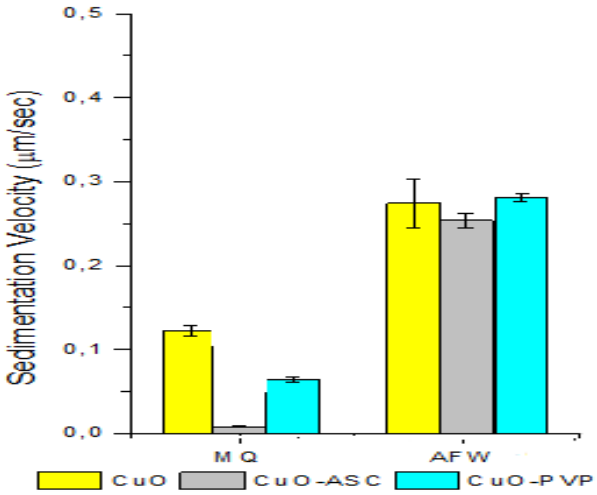

Figure 1-7 Average sedimentation velocity data for pristine and SbyD CuO NMs (ASC and PVP) diluted in Milli-Q and OECD 203 (without PBS). Figure is adapted from Ortelli et al. (2017).

Sedimentation velocity data showed a correlation with the hydrodynamic diameter determined by DLS (Tab. 1-6) for all the samples investigated. In general, it was confirmed that the modifying agents improved the colloidal stability of the dispersions, preventing or decreasing the formation of CuO aggregates with respect to pristine CuO NMs (Ortelli et al. 2017).

As for the pristine CuO NMs, samples were prepared following the same procedure to measure the released ions from the SbyD CuO NMs. Here data reported in table 1-8 were relative to pristine and modified CuO samples diluted in OECD 203 compared with those obtained prior to dilution.

Table 1-8 $Cu_{dissolved}/CuO_{total}$ % of pristine and modified CuO samples diluted in Milli-Q water and OECD 203 medium. *Data acquired from (Ortelli et al. 2017).

	$Cu_{dissolved}/CuO_{total}$ (%)		
	Milli-Q water	**OECD 203 in PBS**	**OECD 203 in Milli-Q**

	(t=24h; T=37°C)	(t=24h; T=25°C)	**water** (t=24h; T=25°C)
Pristine CuO NMs	0.231	0.093	< 0.02
SbyD (PVP) CuO	0.287	0.071	0.1*
SbyD (ASC) CuO	2.488	0.896	0.3*

For the pristine and PVP modified NMs, which had a higher HDD (Tab. 1-6), samples showed the lowest values of dissolved Cu. Interestingly, data gathered from the SbyD (ASC) CuO diluted in OECD medium, showed a much lower release of ions compared with the same NMs in just Milli-Q water, probably due to the formation of complexes with the salts present in the medium.

Finally, to confirm and measure the amount of coating agent adsorbed on the CuO NM surface, a thermal analysis on the dried CuO NMs modified samples was performed at ISTEC-CNR (for details about the methodology see Appendix A, subsection A.5).

Table 1-9 Thermogravimetric results achieved for CuO pristine and modified samples (ultra-filtered (UF) and not ultra-filtered (NO UF)).

		Phosphate + Coating agent		**Coating Agent**	
	Weight loss (%)	Adsorbed Amount (wt %)	Total amount (wt %)	Adsorbed Amount (wt %)	Total amount (wt %)
Pristine CuO NMs	2.6				
$CuO(PO_4)^{3-}$(UF)	3.5	5.6			
$CuO(PO_4)^{3-}$(NO UF)	4.2		9.5		
SbyD-PVP(UF)	6.3	4.3		3.2	
SbyD-PVP(NO UF)	10.1		8.8		7.8
SbyD-ASC(UF)	4.7	2.2		1.3	
SbyD-ASC(NO UF)	9.2		6.8		5.9

Data revealed that after ultrafiltration of the SbyD CuO-ASC dispersion, only 1.3% of the ASC added in solution was absorbed onto the core CuO NM. It is likely, that the negative charge of ASC adversely affected the coating properties of the negatively charged CuO NMs in PBS, determining a % adsorbed capacity calculated as amount of adsorbed in mg/amount of adsorbent used for adsorption expressed in g for ASC of 32% and 49% for PVP, characterised by neutral charge.

The strong influence of the surface charge on the coating agent adsorption is further confirmed by the data found in the analysis of the absorption of ASC in the stock suspensions in Milli-Q water without PBS (data not reported here), where the adsorbed capacity of ASC was 61%, demonstrating the stronger coating performance of ASC on positively charged CuO NMs.

1.2.4.3 Fragmented Product of CuO NMs

NMs may be embedded into polymeric matrices or thin films to form nanocomposite materials, food packaging, or medical devices; any NMs released from these materials will likely have polymeric material attached (Louie et al. 2016). In order to perform ecotoxicological comparative and comprehensive testing, the following would be needed: the matrix without the NMs, the pristine NM and the fragmented product (FP) containing both, to represent the released materials. To obtain samples, close to exposure conditions as expected in environmental settings, weathering (simulation of natural transformations in the environment, *e.g*. exposure to sunlight using Suntest apparatus; http://atlas-mts.com/products/) can be applied to the FP to generate "Weathered Fragmented Products" (WFP). Finally, when these WFPs are tested under environmentally relevant conditions in toxicological or ecotoxicological test media over a specific time frame, they likely undergo further changes resulting in "aged fragmented products" (AFP) (Nowack et al. 2016). Not all these processes were always easy to replicate in the laboratory, resulting in difficulties in producing these materials for toxicity testing.

In order to investigate such materials, pristine CuO NMs were incorporated in products of the SUN industrial partners and subsequently reduced to Fragmented Products (FP), to mimic the use-phase, represented by a form of the matrix with a greatly increased surface area but constituting otherwise an identical material to the one in the real products (Nowack et al. 2016). FP production was performed for the pure matrices (without NMs) as well which served as a reference during further testing (SUN 2014b).

The SUN project partners (BASF and EMPA) encountered several issues when trying to generate sufficient amount of materials necessary to perform ecotoxicological studies.

Therefore, it was possible to produce only small amounts of material, 2.33g for CuO_Acryl_FP and 2.13g for the matrix control, Acryl_FP. Thus, two suspensions were provided:

- 1.17 g Acryl_FP suspended in 73 g Milli-Q water (16g L^{-1} Acryl_FP);
- 1.35g CuO_Acryl_FP (= 20mg CuO) suspended in 64.3 g Milli-Q water (16g L^{-1} Acryl_FP).

Secondary characterisation data provided for these materials were (Nowack et al. 2016):

- ✓ elasticity modulus of matrix (Pa): 10^{-7} (viscoelastic) and
- ✓ median particle size distribution of 73 μm.

Due to the small amount of material obtained, total copper and Cu ions release studies were performed with these materials only dispersed in Gamble's medium (simulated lung fluid) by ISTEC-CNR. This medium is as well composed of mixture of salts has a pH 7.4 similar to the OECD 203 used in these experiments, so some of the findings can be used to interpret the data gathered by the experiments in this research project (see chapter 4). ISTEC-CNR found, interestingly, very low concentrations of Cu ions in the Acryl_FP sample, representative of the matrix without CuO NMs. Furthermore, the concentration of total Cu released from CuO_Acryl_FP was just slightly higher than the ionic fraction detected in the ultra-filtered sample, suggesting a negligible presence of Cu ions in CuO NMs form the total release.

In this research project, the CuO_Acryl_FP amount provided was very low allowing only chronic studies with juveniles of *L. stagnalis*. Given the low % of Cu in these materials, it was considered that investigating the sub-lethal biomolecular response was the most appropriated and sensitive parameter to assessing changes following exposure.

1.3 Model test species: *Lymnaea stagnalis*

Invertebrates are composed of a large and very diverse group of animals, consisting of more than 30 different phyla, several of which include more than 1000 different species (Baun et al. 2008a). These taxa are of extreme importance as test species for the risk assessment of NMs playing an important ecological role in the aquatic ecosystem, where the release of NMs might be taken up by planktonic or benthic invertebrates through different exposure routes (*e.g.* via direct uptake from the water phase or through food/ingestion) (Baun et al. 2008a).

Despite their importance and recognition of the threat that pollution poses on mollusc populations, freshwater molluscs have long been underrepresented in toxicity databases used to develop water-quality standards, where the inclusion of toxicity data on molluscs is not required in either the USA or Europe. Nevertheless a vast amount of toxicity data for freshwater mussels have been published, which have been found to be highly sensitive to some aquatic contaminants (*e.g.* metals and ammonia), but the availability of laboratory toxicity data for freshwater snails remains limited (Besser et al. 2016). Most of the work reported concerns acute tests on adult snails, with a limited amount on embryo and hatchling snails.

This research project focussed on the assessment of the exposure of the benthic ecosystem, represented by the common pond snail *L. stagnalis* (Fig. 1-8), chosen as a representative species of the environment, to CuO NMs and associated products.

Figure 1-8 Adults of *L. stagnalis* reared at Heriot-Watt university laboratories.

Pulmonata freshwater snails of the genus *Lymnaea* are found in lentic systems, where they play an essential role in the consumption and decomposition of aquatic plants and epiphyton (Ruppert et al. 2004). *L. stagnalis*, due to its biological features, such as rapid growth, short generation time, and high reproductive output, has been used as model species in experimental research in the field of neuro-biology, embryology, biochemistry, parasitology and toxicology (Arambaši et al. 2013). Indeed, *L. stagnalis* is considered a good biomonitor for metal pollution given its capacity to accumulate metals, its suitable size for metal analysis and its wide distribution (Croteau and Luoma 2007).

Studies have shown that although relatively tolerant to acute metal exposure, freshwater pulmonata snails are amongst the most sensitive aquatic organism to chronic exposure to at least some metals (Ng et al. 2011). Depending on species, either mortality or growth seems to be the most sensitive endpoints tested to date for chronic exposure to several metals (Co, Pb, and Ni). For example, *L. stagnalis* exhibit greatly reduced growth at 16 gL^{-1} dissolved Pb during a 30-day exposure which makes it one of the most sensitive species tested (Grosell et al. 2002, Brix et al. 2011).

Notwithstanding, very little is still known about the potential biological accumulation of CuO NMs in *L. stagnalis*, since only these 2 studies (Misra et al. 2012a, Croteau et al. 2014b) have been published on the topic. Investigations of the lethal toxicity of CuO NMs on *L. stagnalis* have not been reported. However, Ali and Ali (2015) examined the induction of oxidative stress and DNA damage following at5 days waterborne exposure to pristine CuO NMs in the snail *Lymnaea luteola L.*. Activation of antioxidant enzymes was found at the low concentration of 7 μgL^{-1}, and DNA impairment was observed in digestive gland cells with increasing CuO NMs concentration and exposure time.

In general, most of the data concerning toxicity of copper to *L. stagnalis* were derived from studies using the ionic form of copper. Brix et al. (2011) studied the toxicity of ionic Cu as $CuSO_4$ on hatchlings of *L. stagnalis*. They attributed the acute sensitivity of hatchlings to Cu^{2+}, with an estimated $LC50_{96h}$ 30.7 μgL^{-1} Cu, to the evolved mechanism of withdrawal of these snails into their shells. This behaviour is associated with a high Na^+ loss with haemolymph expulsion that *L. stagnalis* is normally capable of sustaining, which thus may have led to *L. stagnalis* being particularly resistant to acute Cu^{2+}-induced Na^+ loss (Brix et al. 2011).

In contrast, Cu-induced loss of 30% extracellular Na^+ is considered a lethal threshold in fish. Copper taken up by fish gills inhibits Na^+/K^+-ATPase, which causes osmoregulatory disturbance, as Na^+ is lost (Laurén and McDonald 1986). To make up for Na^+ losses, fish might increase the speed of his influx, which led also to a faster be Cu^{2+} influx given the ability of Cu to cross membranes using the Na^+ transport system (Croteau and Luoma 2007).

Comparable lethal concentrations ($LC50_{96h}$, $LC20_{96h}$: 24.9, 18.0 μgL^{-1}) values were estimated in the acute study of Ng et al. (2011) with young adults (1.9–2.3 cm) of *L. stagnalis* exposed to a range of nominal concentrations of Cu between 0 and 100 μgL^{-1}.Tissue concentrations of Na and Ca were also reduced by Cu in the acute exposure. Chronic experiments were also performed in this study. Snails were exposed to lower concentrations of Cu (0, 5, 10, 20 and 35 μgL^{-1}) than in the acute tests. During a 28d, chronic exposure in the presence of food there was no significant mortality but an inhibition of growth was observed. These authors attribute the difference in the toxicity to Cu^{2+} detoxification mechanisms evidenced by increases in metallothionein-like protein concentrations and Cu^{2+} binding to metal-rich granules, lipid peroxidation, and changes in the subcellular distribution in the soft tissues (Ng et al. 2011).

More recently, Atli and Grosell (2016) measured the enzymatic and non-enzymatic responses to ionic Cu exposure as $CuSO_4$ (2– 90 μgL^{-1} for 48 h) in different tissues of adult of *L. stagnalis*. The hepatopancreas was affected by Cu exposure, even at the lowest concentration, where antioxidant enzymes, glutathione peroxidase (GPX) and catalase (CAT) were activated. Thus, authors suggested that antioxidants activity may be responsible for the high tolerance to acute exposure metal observed in observed in adult *L. stagnalis* and could be a suitable biomarker to evaluate the metal exposure and toxicity in the aquatic environment even in relatively low level short term exposures.

Recently, in 2016, *L. stagnalis* was approved as a model species for testing the reproductive toxicity of some chemicals in aquatic molluscs (OECD 2016). Evaluation

for the approval of these standard guidelines commenced two years earlier with the work of Ducrot et al. (2014). This paper provided the scientific basic for the chronic reprotoxicity tests performed in this research project (see Chapter 3), allowing the evaluation of the sublethal toxicity of CuO NMs, the main subject of this study.

L. stagnalis are easily cultured in the laboratory, and due to their rapid growth and short generation times, make determination of reproductive end points practically easier to determine than other species (Besser et al. 2016).

In this research project, *L. stagnalis* was cultured in OECD 203 medium prepared following OECD (1992) protocol, in a pH at around 7.5, water hardness of 250 mgL^{-1} $CaCO_3$ and ammonia below 2 mgL^{-1}.

Snails were kept at room temperature (≈ 20 ˚C) in aerated 9 L (L395 x W255 x D155 mm) plastic tanks at density of 5 snails per litre, with snails separated per size shell. Although *L. stagnalis* is an omnivore species, feeding consisted in mainly lettuce leaves *ad libitum* to avoid rising of ammonia concentration, which occurs very easily when feeding with fish flakes. However, if adult snails were needed, a little quantity of fish flakes *ad libitum* was added to the normal feeding to support growth of juveniles.

One third of water change per tank was performed twice per week, egg clutches (Fig. 1-9) removed while changing the water, in order to have cohorts of snails with similar age, and approximately similar size which facilitates the experiments set up where homogeneous snails' size is very important.

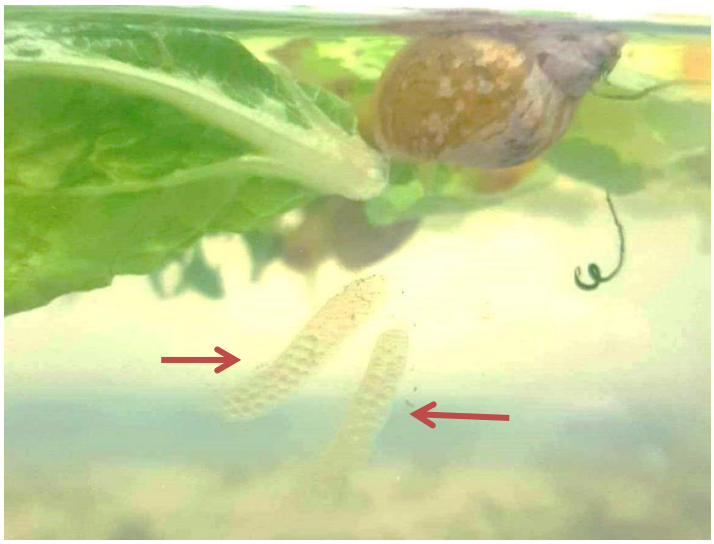

Figure 1-9 *L. stagnalis* and egg clutches. Arrows indicate egg clutches laid on the tank's wall.

Arambaši (1987) determined the two growth stages of *L. stagnalis* from postembryonic to the adult phase. Two parameters were evaluated: the longitudinal growth, defined as the change in the longitudinal growth of the shell; and the weight growth defined as the

absolute weight change of the whole body (Arambaši et al. 1987). In the first stage of development, both parameters increased constantly until sexual mature age was reached at around 2 cm of length. Usually, at 20 ˚C, snails reached this size in about 3 months; however, variation of this time line was observed with changing temperature, feeding, snail density and water quality during culturing. In the second stage symmetrical length growth retardation occurred but the rate in weight growth increased well beyond sexual maturity and its maximum was reached only 20 days later (Arambaši et al. 2013).

In this research project, two different life stages of development were chosen for different experiment designs:

- hatchlings (7-9 days old, ≈ 3 mg wet weight (ww)) were used for acute (96h) and chronic (10 days) experiments, where mortality and molecular changes, respectively, were investigated;
- young adults (≈ 22 mm, ≈ 1.2 g (ww)) were used for chronic (30 days) reprotoxicity studies. Parameters such as number of laid egg masses, changes in weight and behaviour and feeding rate were measured throughout the tests.

L. stagnalis is a simultaneous hermaphrodite that can only perform one sexual role at a time during copulation. They are, usually, receptive as females and during copulation they remain immobile (Koene and Ter Maat 2004) to allow the male to insert his penis, after a sequence of well-known movements, and transfer a copious amount of sperm (De Visser et al. 1994). Under the controlled conditions of a laboratory, *L. stagnalis* lays eggs in egg masses (clutches) that it fixes to a substrate regularly (see Fig. 1-9) (Ter Maat et al. 2012). The number of embryos per clutch increases linearly with adult size. At 2 cm size, snails lay approximatively 2 clutches with 40-50 embryos per week; at 3.5 cm, a clutch could contain up to 160 embryos. Embryos hatch after 15 to 30 days at 20°C.

Egg laying can be triggered by increasing the food amount and/or a transfer of the snails to clean water, due to a combination of composition of the water and a clean substrate (Ter Maat et al. 2012). It takes the animal around 2 hrs to build the egg mass internally and subsequently fix it to the substrate (Koene and Ter Maat 2004).

Adults of *L. stagnalis*, as well as other types of gastropods, are particularly suitable models for a variety of neurobiological research, because of their relatively simple and well-studied nervous system (Inoue et al. 1996, Lukowiak and Syed 1999). Researchers have extensively focussed on the study of neuronal mechanisms underlying control of *L. stagnalis* (Inoue et al. 1996, Lukowiak et al. 1996, Lukowiak and Syed 1999, McComb et al. 2005, Byzitter et al. 2012, Lukowiak et al. 2014) respiratory systems, mainly in

order to study the neuronal mechanisms that underlie associative learning, memory formation and its retain (Lukowiak et al. 2006).

L. stagnalis performs bimodal respiration. It is able to exchange gases across their skin (cutaneous respiration) and through its rudimentary lung (aerial respiration) at water surface. Under normoxic conditions, cutaneous respiration predominates (6 ml O_2L^{-1}), while under hypoxic (< 0.1 ml O_2L^{-1}) pond water conditions, aerial respiration predominates (Lukowiak et al. 1996) and respiration rate increases. This allows *Lymnaea* to exploit environmental niches that are not readily available to 'purely' terrestrial or aquatic species (Karnik et al. 2012).

Aerial respiratory behaviour is periodic and the ventilator cycle follows the sequence: expiration, inspiration, respiratory pause. It is easily observable and quantifiable (i.e. number of breaths and duration of breath) and for this reason, it has been used to evaluate the effect of chemicals on the organism (Byzitter et al. 2012). Furthermore, aerial respiration of *L. stagnalis*, can be, also, operantly conditioned and modified both by experience and by environmental factors.

Byzitter et al. (2012) demonstrate that snails acutely exposed (48 hrs) to a combination of heavy metals, at concentrations below those allowable in municipal drinking water (Zn: 1100 μgL^{-1}; Cd: 3 μgL^{-1}), showed behavioural plasticity. Long term memory formation (LTM) was assessed by operant conditioning the respiration behaviour showing that heavy metals have a negative effect on cognition in invertebrates. Aquatic snails rely on learning and memory to assess predation risk and learn to recognise xenogeneic alarm cues activating predatory response behaviours (Dalesman and Rundle 2010). The presence of heavy metals reducing the snail's ability to learn and form memory may therefore impact ultimately their distribution within the habitat (Byzitter et al. 2012).

In this research project, the operant conditioning of the respiration behaviour, and so learning and formation of long-term memory was used as a non-invasive tool to assess the toxicity of CuO NMs (see Chapter 4). Recently, in 2017, a study was published applying this method to evaluate the toxicity of Ag NMs (Young et al. 2017), validating the sensitivity and the applicability of this endpoint to the risk assessment of NMs. Young et al. (2017) found Ag NMs and $AgNO_3$ blocked learning and memory formation at concentrations of 50 μgL^{-1} and 10 μgL^{-1}, respectively. They attributed the memory deficits observed in snails exposed to $AgNO_3$ to induced oxidative stress, instead they speculated that Ag NMs act as an irritant on a sensory structure (the

osphradium), causing a stressful response similar to other stressors (*e.g.* thermal) that alters the snails' ability to form memory.

1.4 General objectives of the research

This research project was developed within WP-4 of the European FP7 project SUN "Sustainable Nanotechnologies" which aimed to address the entire lifecycle of nano-enabled products. The NMs released from these products in different lifecycle stages were gathered during the project to characterize their physico-chemical properties for the purposes of (eco) toxicity testing and behaviour/fate experiments and to compare the results with the pristine NMs. To this end, a specific case focussed on CuO NMs was selected based on their use in the market, their representativeness of the whole classes of metallic NMs and results from gap-analysis from other projects and literature. In order to address the main goals dictated in the SUN project, this research project had the overall aim of evaluate the ecotoxicology of CuO NMs on the benthic ecosystem. This aim was addressed via the objectives stated below:

- Investigation of the acute lethal toxicity of CuO NMs using the invertebrate snail *Lymnaea stagnalis*, under the influence of multiple stressors, to estimate sublethal concentrations to perform chronic exposures;
- Performance of long-term experiments using different life stages of *L. stagnalis* to assess toxicity and secondary effects on reproduction, behaviour and expression of metallothionein and key antioxidant enzyme systems;
- Performance of short and long-term ecotoxicity tests using modified CuO NMs and fragmented products (FP) containing the pristine CuO NMs.

In the following Chapters, the experimental work performed during the 3 years of this PhD is presented. Acute and chronic lethal and sublethal toxic effects of CuO NMs on different life stages of *L. stagnalis* and the corresponding mechanisms including oxidative stress, mortality, reproduction, growth cognitive impairment are assessed.

Initially, in Chapter 2, acute lethal toxicity of the pristine and SbyD CuO NMs, as well as the ionic form of Cu, was investigated as background for the following chronic experiments. Different life stages of *L. stagnalis* have proven to have different sensitivity to contaminants (Niyogi et al. 2014). Thus, chronic toxicity studies are presented in Chapter 3 and 4 where young adult snails were used to evaluate the effect of the CuO NMs on reproduction, growth, feeding rates and behaviour. In Chapter 5, chronic toxicity studies are reported where juveniles of *L. stagnalis* were used to assess

the impact of the different CuO NMs on the antioxidant system of the snails through changes in gene expression of key enzymes.

Chapter 2 Acute toxicity of pristine and SbyD CuO NMs on juveniles of the snail, *L. stagnalis*

2.1 Introduction

Metal-based engineered NMs (Me-NMs), like copper oxide (CuO) NMs, have been, in the last decade, increasingly utilised due to their particular physico-chemical properties that allow their use in new industrial applications and consumer goods. The escalation in the manufacture of Me-NMs results in their increased release into the environment during the different product's life-stages (Nowack et al. 2012b, Ramskov et al. 2015, Nowack et al. 2016). For instance, CuO NMs, due their numerous applications in antimicrobial products, such as wood preservation, antimicrobial textiles, agricultural biocides and antifouling paints, are expected to enter the aquatic environment via direct discharge *(e.g.* dissolution of antifouling paint applied on hull's boats) or run-offs from wastewater treatment plants (Ramskov et al. 2015, Hou et al. 2017a). Studies have shown that most of the Me-NMs tend to agglomerate and deposit on the sediment in seawater and freshwater ecosystems (Buffet et al. 2013, Ortelli et al. 2017) posing a high biological risk especially for benthic organisms, which make up a large proportion of bottom habitat in fresh and seawater environments. Further biological, chemical and physical mechanisms (*e.g.* interaction with other chemicals and sediments. bacterial decomposition or bioturbation) also occur at the sediment level (Hanna et al. 2013) transforming these materials and their potential toxicity to the organisms (Ramskov et al. 2015).

In recent years, with the aim of controlling NMs' risk maintaining their physical-chemical properties in the environment, safer by design (SbyD) solutions have been proposed by functionalising the surface of NMs with engineered macromolecular coatings (Louie et al. 2016). Coatings, such as molecules (*e.g.* ascorbate (ASC)) and polymers (*e.g.* polyvinylpyrrolidone (PVP)), are often used to prevent the natural tendency of NMs to agglomerate in the aquatic environment. Indeed, in absence of coating, NMs' behaviour is mostly governed by their core intrinsic properties such as size, zeta potential, and medium ionic strength (Miseljic and Olsen 2014). Surface functionalisations are most likely to have a high relevance for exposure, toxicity, and risk assessment of NMs; thus, it is important to compare pristine with modified NMs. In addition to stabilizing the NMs in the medium, surface changes could influence the percentage of released ions from the core NM and facilitate entry of the NMs into the cells influencing their uptake pathways (Croteau et al. 2011b). Nevertheless the influence on bioavailability and toxicity of coatings is not well fully explored to date (Croteau et al. 2011a).

Several studies have been published recently reporting on the influence of coatings in the toxicity of NMs on freshwater organisms (Tejamaya et al. 2012, Baumann et al. 2014, Oliver et al. 2014, Stoiber et al. 2015). These studies indicate that the increased stability of NMs in the medium likely contributes to the effects on the exposed organisms; however, results are very varied due to the interaction as well with other chemicals and sediments. For instance, Baumann et al. (2014) exposed 96h neonates of *Daphnia magna* to iron NMs functionalised with different coating agents, among which ASC and PVP, by thermal decomposition and reduction of iron (III) acetylacetonate in diethylene glycol (DEG). Acute toxicity was different for each coating agent and toxic effects could not been correlated with the change of hydrodynamic diameter observed in the coated NMs nor with the type of stabilizing forces (steric or electrostatic). Effects were instead linked to decreasing colloidal stability, the release of ions and the ability to form reactive oxygen species (ROS) in the medium (Elendt M7) of the core material.

In the specific case of CuO NMs, very few studies have been published investigating the influence of coating agents on their toxicity (Clar et al. 2016, Javed et al. 2017); in contrast, numerous published studies have assessed the potential toxicity of pristine CuO NMs. These materials have been proven to have detrimental effects on a wide range of aquatic organisms including algae (Perreault et al. 2012, Melegari et al. 2013), molluscs (Pradhan et al. 2012, Croteau et al. 2014b, Ali and Ali 2015), crustaceans (Wu et al. 2017) and fish (Bondarenko et al. 2013, Ates et al. 2015). Toxicity has been attributed to either release of Cu^{2+} ions or to a so called " nanoparticulate effect" (Croteau et al. 2014b).

For instance, Pradhan et al. (2012) evaluating the waterborne and dietary exposure of CuO NMs to the freshwater invertebrate shredder *Allogamus ligonifer*, attributed the sublethal and lethal toxicity observed to the leached ionic copper from CuO NMs. Findings demonstrated negative effects on larval feeding, growth and high amounts of accumulated copper inside the larval body after exposure to the higher concentration of CuO NMs (75 mgL^{-1}) via water or pre-contaminated food.

In contrast, Griffitt et al. (2008) comparing the acute toxicity of soluble copper and CuO NMs suspensions to zebrafish, *Danio rerio*, recorded a very low dissolution of CuO NMs. At 48h, an $LC50_{48h}$ value of 1.5 mgL^{-1} was estimated and different morphological effects and global gene expression patterns in the gill observed could account only for 10–15% of the dissolution of the NM.

In addition, CuO NMs have been reported to produce other mechanisms of toxicity mediated through oxidative stress response (Buffet et al. 2013, Mouneyrac et al. 2014),

DNA damage (Gomes et al. 2012) and bioaccumulation (Croteau et al. 2014b). As dissolution is not the only factor explaining NMs toxicity, adverse effects following CuO NMs exposure have been suggested to be caused by particle-specific effects (Bondarenko et al. 2013, Ivask et al. 2014). As described previously, the size, shape, composition, aggregation and solubility of NMs (metal-based NMs) may be related to their toxicity (Rossetto et al. 2014). Overall, in these studies CuO NMs demonstrated stronger adverse effects than the bulk counterpart CuO demonstrate, but always lower than the ionic form of Cu. Furthermore, different studies have confirmed that the presence of natural organic matter (NOM) reduce the toxicity of CuO NMs (Blinova et al. 2010) highlighting the likely difference in the toxicity of these materials between the laboratory experimental setting and the real environment.

In light of this, in the present study, a comparative assessment of the acute lethal toxicity of modified (ASC and PVP) and unmodified CuO NMs and the ionic form of Cu as $CuSO_4$ on the juveniles (≈ 7 days old) of the freshwater gastropod, *L. stagnalis*, is reported.

L. stagnalis is used as model species of the freshwater benthic ecosystem. Freshwater gastropods being important ecosystem engineers can alter, create and significantly modify a habitat. They provide essential ecosystem services by accelerating detrital decomposition, releasing bound nutrients into solution by their feeding activities, excretion, and burrowing into sediments. Changes in the abundance of these taxa could affect directly or indirectly the entire ecosystem (Covich et al. 1999, Byzitter et al. 2012). Sediment is recognized as a major environmental sink for contaminants, including Me-NMs. Consequently, sediment-living organisms, like snails, are likely to be the most at risk for exposure to these materials (Ramskov et al. 2015).

Although relative resistant to metal pollution in the adult stage life, juveniles (from 0 to 7 days old) of *L. stagnalis* have been regarded as one of the most sensitive species to pollution from metals, such as Ni, Pb, Co and Cu (De Schamphelaere et al. 2008, Grosell and Brix 2009, Brix et al. 2011, Niyogi et al. 2014). In long-term experiments snails growth emerged to be the most sensitive endpoint as a result of their high calcium requirement which facilitates the uptake of the metals into the soft tissues (Gao et al. 2017).

Investigations of the lethal toxicity of CuO NMs on *L. stagnalis* have not been reported to date and very little is still known about the potential biological accumulation of CuO NMs in *L. stagnalis*, since only two studies have been published so far. In these studies, young adults of *L. stagnalis* were exposed to environmentally relevant concentrations of

CuO NMs, thus the use of isotopically modified CuO NMs (^{65}CuO NMs) was necessary to distinguish the uptaken Cu from the already high background of Cu (34 $\mu g g^{-1}$) in *L. stagnalis* (Misra et al. 2012a). Both studies indicated an insignificant influence of the dissolution of Cu from the CuO NMs to the high percentage (80-90 %) of the bioaccumulated Cu (Misra et al. 2012a, Croteau et al. 2014b).

Finally, no toxicity studies exist that have investigated the toxicity of the transformed CuO NMs due to interaction with the environment in which they are released. As previously stated, organisms will be most likely exposed to this form of NMs instead of the pristine form. Hence, the toxicity that these NMs may display will require careful evaluation and testing to ensure effects can clearly be attributed to the causal factors (Kumar et al. 2012).

The aim of this study was to determine the effectiveness of the SbyD solutions adopted to modify the pristine CuO NMs, comparing the potential acute lethal toxicity of these materials to the sensitive juvenile stage of *L. stagnalis*. The present study is the first investigation of the toxicity of CuO NMs, including modified NMs, with the aim of reducing their effects, to the model gastropod *L. stagnalis*.

2.2 Materials and Methods

2.2.1 Test chemicals and NMs characterisation

Pristine CuO NMs (nominal size 20nm) were provided in powder by PlasmaChem GmbH© (within the SUN project consortium) and $CuSO_4*5H_2O$ (analytical reagent grade) was purchased from BDH Prolabo®.

$CuSO_4$ stock solution was prepared, immediately before use by dissolving the appropriate amount of copper sulphate in Milli-Q water (Millipore, Livingston, UK) with a grade of 18.2 MΩ cm.

To prepare the pristine CuO NMs suspension, the required amount of CuO powder was suspended in 100 ml of Milli-Q water and then sonicated at 38 kHz ± 10 % in a bath sonicator (Pulsatron 325, Kerry) for 8 minutes twice, with ~10 seconds manual shaking in between, following the protocol of Jacobsen et al. (2010).

Stock suspensions of SbyD CuO NMs coated with coating agents PVP, which provide steric stabilisation, and ASC, which stabilize via electrostatic repulsion, were synthesised and provided by ISTEC-CNR, Faenza (IT) (within the SUN project consortium) at the concentration of 10 gL^{-1} Cu. To prepare working stock suspensions,

the provided stocks were vortexed thoroughly before dilution (1:100) in the culturing medium, OECD 203 (OECD 1992).

In either case, stocks were continuously stirred while the necessary amount was aliquoted out to achieve the desired concentration in the exposure vessel.

SbyD NMs were synthetized using two different dispersion media: Milli-Q water and PBS. Thus, four different SbyD CuO NMs were tested, namely: CuO-PVP(PO_4^{3-}) NMs, CuO-ASC(PO_4^{3-}) NMs, CuO-PVP(H_2O) NMs and CuO-ASC(H_2O) NMs.

Furthermore, to distinguish the influence of PBS on the mechanisms of actions of the coating agents, pristine CuO NMs dispersed in PBS were also produced by ISTEC-CNR, namely as: pristine CuO (PO_4^{3-}) NMs (pristine CuO in PBS).

Nanomaterials' characterisation was performed by the University of Venice and ISTEC-CNR as part of the SUN project consortium, as previously described.

Dynamic light scattering (DLS, ZetaSizer nano ZSP, Malvern Instruments, UK) was used to measure hydrodynamic diameter (d_{DLS}) and zeta potential (ζ-pot_{ELS}) measurements were performed by electrophoretic light scattering (ELS). Measurements of all the NMs either in Milli-Q water or in the culturing medium, OECD 203, were performed in triplicate at a concentration of 10 gL^{-1}. Transmission electron microscopy (TEM) images were obtained only for pristine CuO NMs in Milli-Q water.

Furthermore, velocity of sedimentation analyses were performed for the SbyD NMs synthetized in Milli-Q water from centrifugal separation analysis (CSA), using the multi-wavelength dispersion analyser LUMiSizer 651® based on STEP™ technology.

ICP-OES analysis on samples prepared using ultracentrifugation methods at a concentration of 50 mgL^{-1}, were performed to calculate the dissolution of Cu ions from all the CuO NMs in Milli-Q water and OECD medium.

Finally, to verify the amount of coating agent adsorbed on the surface of the SbyD CuO NMs, thermal analyses were performed on dried SbyD CuO NMs samples obtained after ultrafiltration of the stock suspensions at concentration of 10 gL^{-1} Cu.

2.2.2 Test organisms

Early life stages of *L. stagnalis* have been demonstrated to be a sensitive bioindicator capable of detecting even very low levels of environmental stressors (Mazur et al. 2016). The ease of breeding in laboratory, high reproduction, and large amount of embryonic and juvenile forms for the tests allows the statistical assessment of toxic effects in large groups for this development stage. The test procedures for acute experiments are simple and inexpensive and can be performed in battery given the high

reproduction rate of laboratory cultures. Toxic effects can also be detected as changes in the surface morphology of *L. stagnalis* which can easily be observed microscopically (Mazur et al. 2016).

L. stagnalis used within this study were from in-bred laboratory culture. Snails were reared at room temperature and with natural light cycle in 10L tanks of freshly prepared OECD 203 medium, consisting of: 79 mgL^{-1} Ca^{2+}, 38 mgL^{-1} Mg^{2+}, 12 mgL^{-1} Na^{+}, 17 mgL^{-1} and 2 mgL^{-1} K^{+} with a pH of 7.7. General water hardness was around 250 mgL^{-1} $CaCO_3$. Snails were fed *at libitum* with romaine lettuce pre-rinsed in the culturing medium. In order to have snails of the same age, freshly laid egg clutches were removed daily and placed in another tank, facilitating the simultaneous hatch of the embryos. Hatchlings were randomly selected and after 7 days (snail's weight ≈ 1 mg ww) placed in the exposure vessels, where they were not fed for 24h prior the start of the experiment.

2.2.3 Experimental design

An acute exposure experimental design was developed following the protocol of Brix et al. (2011). In brief, juvenile snails (≈ 7 days old) were placed in 35 ml polypropylene containers with 30 ml of test medium; three replicates of 15 snails were tested for each exposure concentration. Containers' lids were perforated before starting the experiment to facilitate oxygen exchanges. Static aqueous exposure tests were conducted to determine the concentration–response curves for lethality over 96 hrs to soluble copper as $CuSO_4$, pristine CuO NMs and SbyD CuO NMs. Exposure was performed in a controlled light (16h d/8 hrs n) and temperature (20 °C) environment. Snails were acclimated 24 hrs prior exposure to the exposure vessel to allow the snail to recover from handling-stress resulting in possible bias artefacts. Mortality was noted daily by assessing heart beat via microscopy and recording mortality when no heart beat was detected.

All exposures were based on mass of copper added; dosing was accomplished by adding the desired aliquot from the stocks into the exposure vessels. The concentration ranges for each treatment were selected based on their potential to induce a lethal effect of Cu either in the aqueous or nano-sized form, allowing the plotting of logistic curves from which LC50 values (the concentration required to cause 50% mortality in the population after 96h) could be estimated. After exposure to CuO-ASC(PO_4^{3-}) NMs it was not possible to estimate the LC50, thus only an LC20 at 96h could be calculated.

Nominal test's concentrations ranges, derived from previously conducted pilot studies, were between 0-50 μgL^{-1} Cu for $CuSO_4$, 0-5000 μgL^{-1} Cu for pristine CuO NMs and 0-3000 μgL^{-1} Cu for all SbyD CuO NMs investigated.

Experiments were initially performed to determine if toxicity of pristine CuO NMs differed from that caused by soluble copper; subsequently tests proceeded evaluating if the presence of the coating agents would mitigate toxicity recorded in the exposure with the pristine CuO NMs. Finally, in order to assess if the dispersion media used to synthetises the SbyD CuO NMs would influence toxicity, SbyD CuO NMs suspended in two different dispersion media (Milli-Q water or PBS) were tested. A parallel comparison was also carried out with soluble copper ions to distinguish between the role of free ions and that of the CuO NMs.

2.2.4 Data analyses

SigmaPlot© version 13.0 (Systat Software, Inc.) was used to perform all statistical analyses. All data were checked for normality of distribution Shapiro–Wilk test and homogeneity of variance. Logistic regression analyses followed by one-way analysis of variance (repeated measurement ANOVA (RMANOVA)) were used to establish significant differences among time points within the same exposure scenario. Differences between different exposure experiments were also analysed using analysis of variance (ANOVA) with Tukey's post-hoc analysis. In case data did not pass the Shapiro–Wilk normality test and the Levene's homogeneity of variance test, the non-parametric Kruskal–Wallis test (one-way ANOVA on ranks) was used and pairwise comparisons were performed using the Dunn's test with a Bonferroni correction for multiple comparisons. All differences were considered statistically significant at $p < .05$. Lethal concentration values (LC50) resulting in mortality of 50% of the population were calculated using a non-linear allosteric decay function designed Microsoft Excel (ToxCalcMix) (Barata et al. 2006).

2.3 Results

2.3.1 Characterisation of CuO NMs

The morphological characterisation of pristine CuO NMs by TEM analysis showed the presence of a spherical NM with a primary average diameter of 12 nm (3min- 35max) (Fig. 1-5). Hydrodynamic diameters' (HDD) data of the pristine NMs, characterised either in Milli-Q water or in the OECD 203 medium, were 1093 (± 50) nm for CuO NMs in Milli-Q water and 2364 (± 282) nm and 2558 ± 472 nm for pristine CuO NMs characterised in OECD 203 medium synthetized respectively in Milli-Q water or in PBS. Data showed an increase in colloidal instability in the OECD medium due to the

presence of the salts which induce agglomeration. The same trend was recorded for the SbyD CuO NMs; however, the presence of the coating agents seemed to mitigate agglomeration of the NMs, particularly for those modified with ASC. Indeed, SbyD CuO-ASC NMs just diluted in Milli-Q had a HDD 122 (± 1.4) nm which is about 9-fold lower that the HDD (1093 ± 50) of the pristine CuO NMs. The same effect was recorded for the NMs diluted in the OECD medium, however with smaller differences (HDD: CuO NMs= 2558 ±472; CuO-ASC(H_2O) NMs= 1293 ±278).

HDD data were confirmed by the sedimented velocity distribution analyses (Fig. 1-7), where, in samples functionalised in Milli-Q water, the coating agents improved the colloidal stability of the suspension resulting on a slowest sedimentation velocity compared to the pristine CuO NMs. However, once introduced in the OECD 203 medium the stability effect was no longer significant (Fig. 1-7).

The average z-potentials (ζ-pot_{ELS}) were for all the NMs investigated within the range of instability (+30 mV> NMs mV> -30 mV) (see Tab. 1-3 par. 1.2.4 for details). The presence of the coating agents did not enhance stability of the materials in either media. To note is the change in charge, once diluted in OECD medium, of the CuO-PVP(H_2O) NMs (ζ-pot_{ELS}= +1.6 ± 0.3 mV) compared to the pristine CuO NMs (ζ-pot_{ELS}= -4.36 ± 0.57 mV).

The mean percentage of dissolved Cu ($Cu_{dissolved}/CuO_{total}$ (%)) in the OECD 203 medium was the lowest for all the materials compared to those dissolved in Milli-Q water (see Tab. 1-3 par. 1.2.4 for details). Especially a strong decrease in dissolution was measured for the SbyD CuO-ASC NMs samples. Indeed, samples diluted in Milli-Q water were found to have dissolution of 2.5% which was reduced to 0.9 and 0.3 % for samples diluted in OECD medium, functionalised, respectively, in PBS or Milli-Q water.

Finally, thermogravimetric analyses demonstrated that the negative charge of ascorbate adversely affected the coating properties of the negatively charged CuO NMs functionalised in PBS. Adsorbed capacity percentage calculated for ASC was 32% compared with 61% for samples dispersed in Milli-Q water.

2.3.2 Acute toxicity of aqueous Cu and CuO NMs

A strong concentration-response and time dependant relationship was observed for the 96 hours exposure for all the NMs investigated.

Results showed higher toxicity of Cu in the ionic form as $CuSO_4$ compared to the pristine form of CuO NMs, dispersed either in PBS or in Milli-Q water. Furthermore, it was observed (not quantified) that after exposure to $CuSO_4$, at low concentrations (3.5-6

μgL^{-1} Cu) within the first time point (24 hrs), snails withdrew inside the shell and totally lost motility (Fig. 2-1), nevertheless they were still alive for the rest of the experiment; indicating that immobilization could be a more sensitive endpoint than mortality.

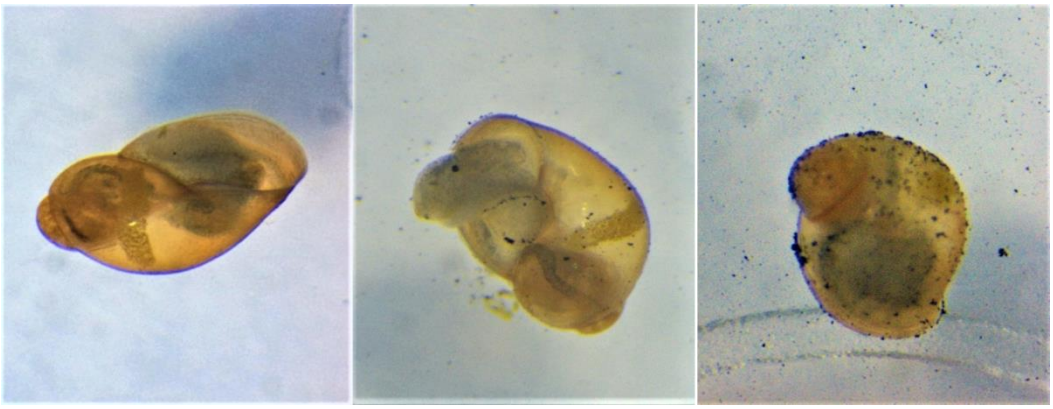

Figure 2-1 Pictures taken with a dissection microscope after acute the lethal experiments. On the left, a dead snail exposed to 10 μgL^{-1} Cu of $CuSO_4$ withdrawn in the shell. On the centre and right, live snails exposed to 3000 μgL^{-1} Cu and 5000 μgL^{-1} Cu, respectively, of pristine CuO NMs grazing on the nanomaterial

LC50 values at 96h were 5.6 (± 0.23 SE) μgL^{-1} Cu, 245 (± 22.7 SE) μgL^{-1} Cu and 2198 (± 143.8 SE) μgL^{-1} Cu, respectively, for $CuSO_4$ (Fig. 2-2) and pristine CuO NMs in PBS (Fig. 2-3) and in Milli-Q water (Fig. 2-4). These findings highlight that the presence of the PBS as dispersion medium increased almost 10-fold the toxicity of the pristine CuO NMs. Indeed, at 96h, percentage of mortality of snails exposed to CuO NMs dispersed in Milli-Q water reached 80% (Fig. 2-4) at the highest concentration (5000 μgL^{-1} Cu), whereas exposure to $CuO(PO_4^{-3})$ NMs (Fig. 2-3) resulted in 100% mortality at a lower concentration of 695 μgL^{-1} Cu.

Statistical comparison of time points, within the same exposure scenario, revealed significant differences (RMANOVA, $p < 0.05$) between the 24 and 96 hrs after exposure to Cu in either aqueous or nano form.

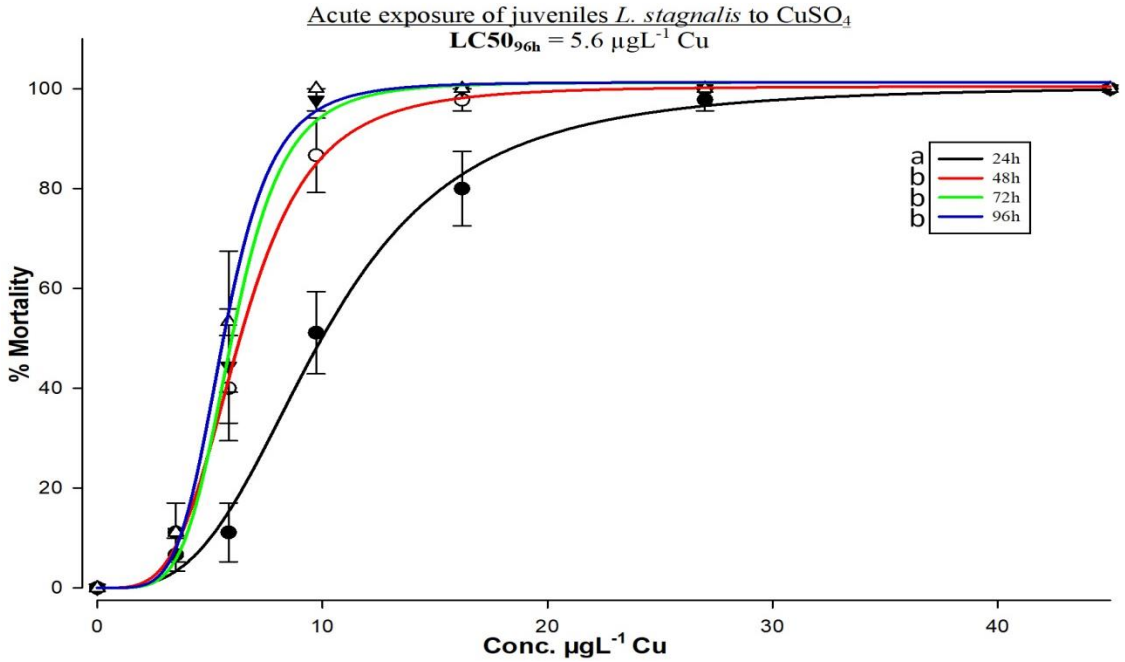

Figure 2-2 Percentage mortality (%) of juveniles of *L. stagnalis* (≈ 7 days old) exposed to increasing concentrations (log. scale 0.7) of Cu as $CuSO_4*5H_20$ at 20 °C for 24, 48, 72 and 96 hrs (n = 3; error bars are SEM). Solid lines stand for the fitted 4-parameters logistic (4PL) model; different letters indicate significant difference ($p < 0.05$) between the different time points to 24 hrs of exposure.

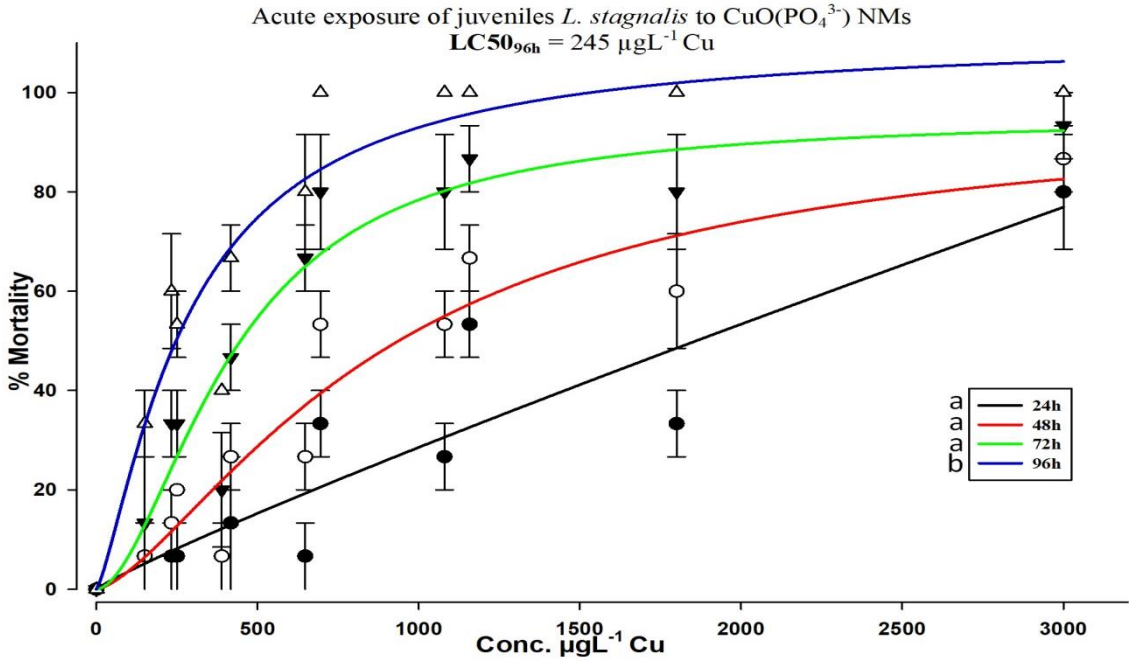

Figure 2-3 Percentage mortality (%) of juveniles of *L. stagnalis* (≈ 7 days old) exposed to increasing concentrations (log. scale 0.6) of Cu as $CuO(PO_4^{-3})$ NMs at 20 °C for 24, 48, 72 and 96 hrs (n = 3; error bars are SEM). Solid lines stand for the fitted 4PL model; different letters indicate significant difference ($p < 0.05$) between the different time points to 24 hrs of exposure.

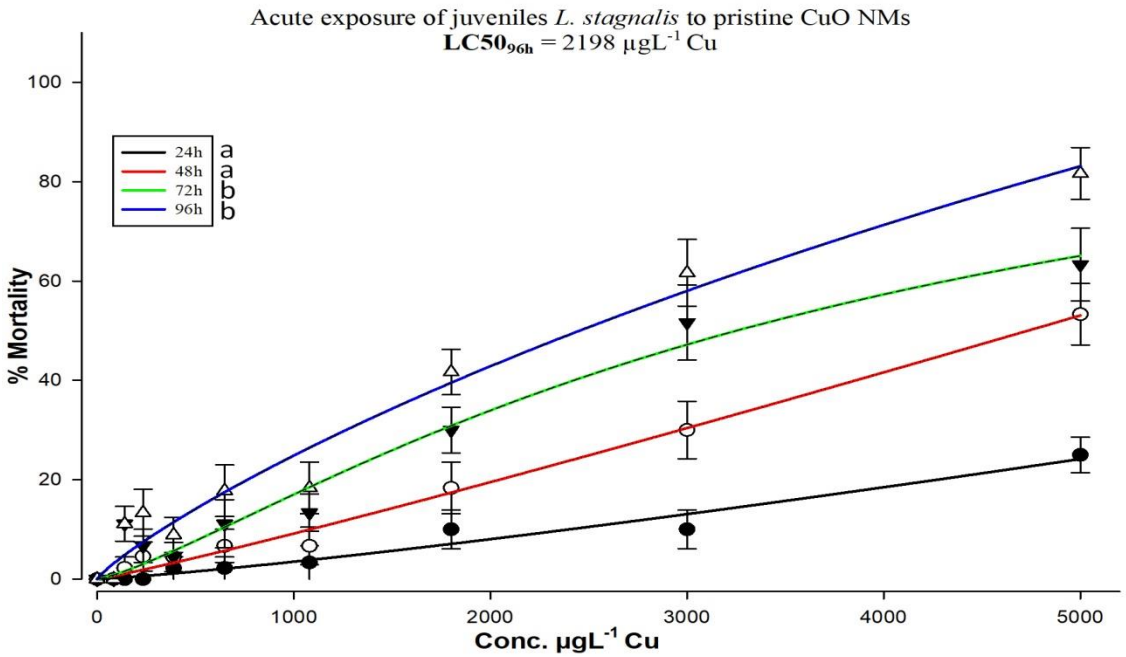

Figure 2-4 Percentage mortality (%) of juveniles of *L. stagnalis* (≈ 7 days old) exposed to increasing concentrations (log. scale 0.6) of Cu as pristine CuO NMs dispersed in Milli-Q water at 20 ℃ for 24, 48,72 and 96 hours (n = 3; error bars are SEM). Solid lines stand for the fitted 4PL model; different letters indicate significant difference ($p < 0.05$) between the different time points to 24 hrs of exposure.

Exposure to the SbyD CuO NMs showed inconsistent findings between the samples dispersed in Milli-Q water (Fig. 2-5) or PBS (Fig. 2-6), suggesting that the dispersion method influenced greatly the toxicity of the NM. Overall, the presence of the coating agents did not reduce the toxicity of the pristine CuO NMs, except for the CuO-ASC(PO_4^{3-}) NMs. For the estimation of potential effects of the coating agents only (ASC and PVP), 96 h single substance toxicity tests were performed, in triplicate, alongside the SbyD NMs exposure tests. No mortality was recorded at any of the time points for the concentrations tested (data not reported).

Fig. 2-5 shows the percentage mortality at 96 hrs of CuO NMs dispersed in Milli-Q water comparing pristine and SbyD NMs. $LC50_{96h}$ values estimated were 1007 (±117.2 SE) μgL^{-1} Cu and 278 (± 29.6 SE) μgL^{-1} Cu for CuO-PVP (H_2O) and CuO-ASC(H_2O) NMs, respectively. There was a statistically significant difference between groups as determined by one-way ANOVA ($F_{(2,33)} = 5.79$, $p = 0.01$). A pairwise Tukey post hoc test revealed a significant difference of the LC50s at 96h between ASC coated CuO NMs and pristine CuO NMs ($p = .008$). There was no statistically significant difference between either the pristine CuO NMs ($p = 0.69$) and CuO-ASC(H_2O) ($p = 0.07$) and the CuO-PVP(H_2O).

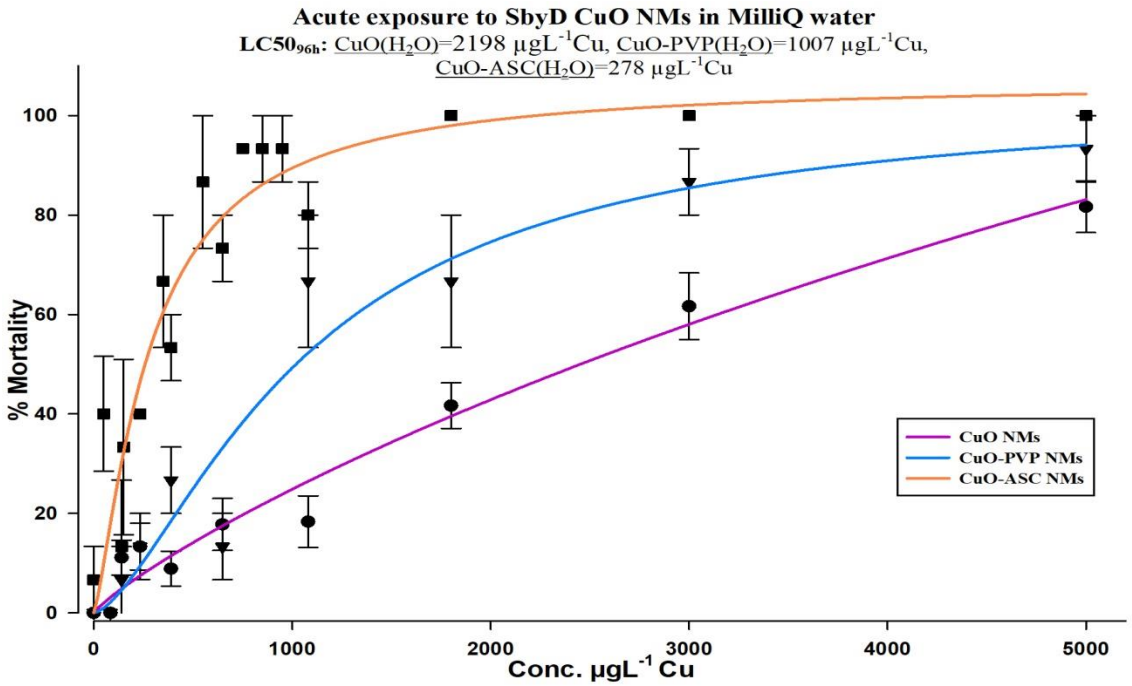

Figure 2-5 Percentage mortality (%) at 96 hrs of juveniles of *L. stagnalis* (≈ 7 days old) exposed at 20 ºC to increasing concentrations (log. Scale 0.6) of CuO NMs functionalised in Milli-Q water: pristine CuO NMs, SbyD CuO-ASC(H_2O) NMs and CuO-PVP(H_2O) NMs. Solid lines stand for the fitted 4PL model (n = 3; error bars are SEM).

Results from the exposure to the SbyD CuO NMs dispersed in PBS are presented in Fig. 2-6. LCx values estimated indicated an interaction between the dispersion medium and the coating agents. Indeed, toxicity of CuO-PVP(PO_4^{3-}) NMs increased greatly compared with the same material functionalised in Milli-Q water (LC50= 1007 ±117.2 SE), resulting in an LC50 value of 171 (±33.3 SE) µgL^{-1} Cu (Fig. 2-5 and 2-6).

Interestingly, in contrast with the other SbyD NMs, after 96h exposure to CuO-ASC(PO_4^{3-}) NMs elicited the hypothesized result of reducing toxicity. LC50$_{96h}$ values could not be estimated within the concentration range tested; thus, lethal concentration value (LC20) of mortality to 20% of the population was estimated at 1471 (± 875.9 SE) µgL^{-1} Cu (Fig. 2-6). Data failed to pass the normality Shapiro-Wilk test, thus a one-way ANOVA on ranks (Kruskal-Wallis H test) was performed. Analysis showed that there was a statistically significant difference in toxicity at 96h between the three NMs ($\chi^2_{(2)}$ = 16.861, $p < 0.001$).

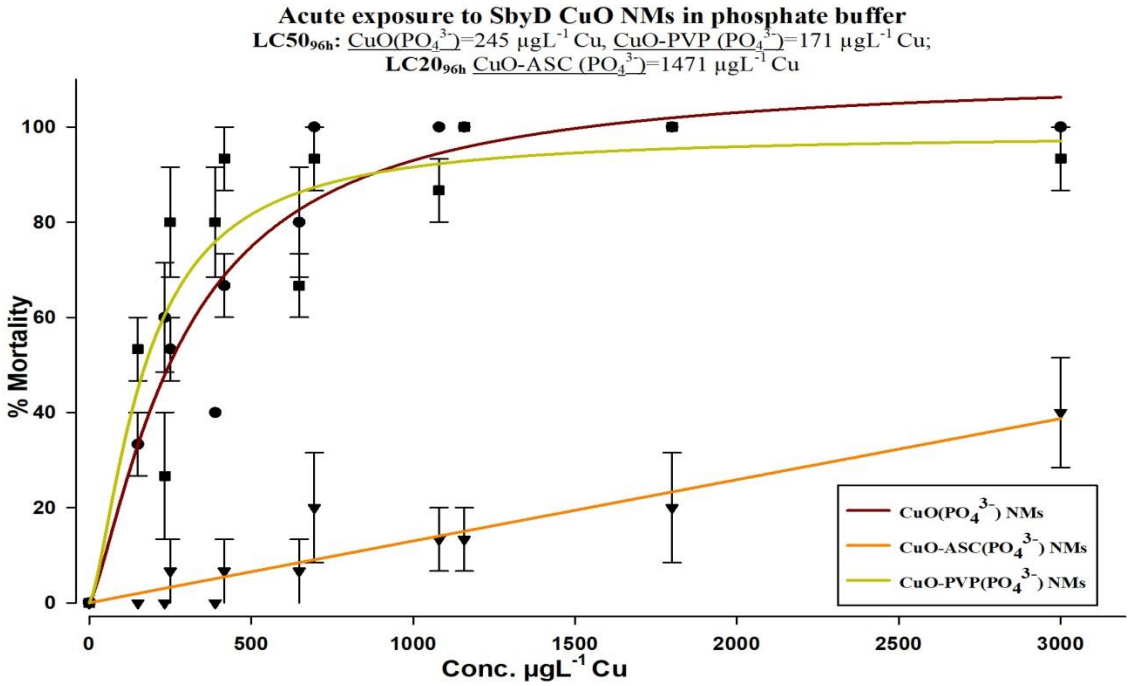

Figure 2-6 Percentage mortality (%) at 96 hrs of juveniles of *L. stagnalis* (≈ 7 days old) exposed at 20 °C to increasing concentrations (log. Scale 0.6) of CuO NMs functionalised in PBS: pristine CuO(PO_4^{3-}) NMs, SbyD CuO-ASC(PO_4^{3-}) and CuO-PVP(PO_4^{3-}) NMs. Solid lines stand for the fitted 4PL model (n = 3; error bars are SEM).

Table 2-1 summarises the results gathered from the acute lethal experiments and the characterization analysis performed on the CuO NMs.

Table 2-1 Summary of the results, LCx values and characterisation data in OECD 203 medium, after acute exposure to ionic Cu and CuO NMs tested (data are means ± SEM).

	LCx_{96h} (µgL^{-1} Cu ± SE)	**d_{DLS} (nm)**	**$Cu_{dissolved}$/ CuO_{total} (%)**	***ζ-pot*$_{ELS}$ (mV)**
$CuSO_4$	LC50 5.6 ±0.23	n/a	n/a	n/a
Pristine CuO NMs (H_2O)	LC50 2198 ±143.8	2558 ± 472	< 0.02	-4.36 ± 0.6
CuO-PVP(H_2O) NMs	LC50 1007 ±117.2	1159 ± 256	0.1	+1.6 ± 0.3
CuO-ASC(H_2O) NMs	LC50 278 ±29.6	1293 ± 278	0.3	−8.1 ± 0.4
CuO(PO_4^{3-}) NMs	LC50 245 ±22.7	2364 ± 282	0.093	-3.37 ± 0.1
CuO-PVP(PO_4^{3-}) NMs	LC50 171 ±33.3	2098 ± 545	0.071	-6.68 ± 0.1
CuO-ASC(PO_4^{3-}) NMs	LC20 1471 ±875.9	1719 ± 157	0.896	-9.52 ± 0.2

2.4 Discussion

The present study addressed the assessment of SbyD CuO NMs' aim to design safer NMs by functionalising them with different coating agents which would aim to reduce

the toxicity of the pristine NMs. A freshwater invertebrate was used as a model species of the benthic ecosystem since it has been demonstrated that this compartment tends to be the environmental end-destination for particulate contaminants, including Me-NMs. A further objective was to test if the dispersant used to synthetize the SbyD NMs would affect their toxicity.

Preliminary experiments were performed to assess and compare the toxicity of Cu of pristine CuO NMs with the ionic form ($CuSO_4$), as baseline for the SbyD studies. The estimated LC50s values obtained for juveniles of *L. stagnalis* suggested little dissolution from pristine CuO NMs given the much greater toxicity recorded following exposures to the Cu salt (Tab. 2-1). These findings agree with the characterisation data, where at 24h hours only 0.02% $Cu_{dissolved}/Cu_{tot}$ was obtained (Tab. 2-1); these findings are supported by many other studies indicating poor dissolution of CuO NMs in the aquatic environment (Griffitt et al. 2008, Buffet et al. 2013, Croteau et al. 2014b). Moreover, during the pilot experiment it was observed that snails, during the 96h exposure, were grazing on the NMs (Fig. 2-1) that once introduced in the exposure medium, were observed to rapidly agglomerate. This was also confirmed by the characterisation data of the pristine CuO NMs, wherein a ζ-potential (-4.36 ± 0.57 mV) and increased HDD (in Milli-Q water: 1093 ± 50 nm; in OECD 203: 2558 ± 472 nm) indicated that this suspension was more unstable when diluted in the OECD medium compared to only Milli-Q water. Furthermore, while dissolution of CuO NMs occurred, it was insufficient to explain the mortality in nano-copper exposures.

Accordingly, Croteau et al. (2014b) investigating the uptake of Cu by *L. stagnalis* from aqueous Cu and CuO NMs trough waterborne and diet-borne exposures, estimated that 80-90 % of the bioaccumulated Cu concentration originated from the ^{65}CuO NMs, indicating that dissolution had a minor effect on Cu uptake from the NMs under their experimental conditions. In particular, a contrast in uptake mechanisms was demonstrated between waterborne and dietary exposure. In the latter, ^{65}Cu uptake from diatoms mixed with ^{65}CuO NMs exceeded uptake from ^{65}Cu-laden diatoms due, mainly, to an increase of feeding rate on the food spiked with the NMs. It appeared that snails were better at detecting (and avoiding) Cu in their diet when diatoms were spiked with ionic Cu indicating dietary uptake as the major route of accumulation of CuO NMs.

The very low $LC50_{96h}$ (5.6 μgL^{-1} Cu) value estimated from exposure to ionic Cu would suggest the snail strain used in this study could be much more sensitive than that in Brix el al. (2011), where, with snails of the same age, a $LC50_{96h}$ of 30.7 μgL^{-1} Cu was derived. This discrepancy could be explained by either different water chemistry of the

experiments medium or by a different genetic tolerance to copper. As previously described, the bioavailability and toxicity of metals to aquatic organisms are dependent on water chemistry factors, thus the same metal concentration in different exposure media induce different toxicity mechanisms (Ng et al. 2011).

For instance, Croteau and Luoma (2007) exposing their organisms through acute waterborne exposures to the isotope ^{65}Cu, demonstrated that the uptake influx for Cu in *L. stagnalis* increased with water hardness. In Brix et al. (2011) water hardness was 123 mgL^{-1} $CaCO_3$ compared with the 250 mgL^{-1} $CaCO_3$ in this study. Additionally, Côte et al. (2015) investigating genetic variation in copper sensitivity of *L. stagnalis*, demonstrated that under copper exposure, mortality varied widely among the eight genetically different populations evaluated. Mortality ranged by a factor of five between the least sensitive population and the most sensitive one (Côte et al. 2015).

Nevertheless, it is likely that mechanisms of toxicity of Cu ions previously demonstrated (Brix et al. 2011, Ng et al. 2011), have played important roles in these experiments. Acute exposure to Cu was attributed to inhibition of Ca^{2+} and Na^{2+} uptake, which being essential and highly required for snails at this development stage (Ebanks and Grosell 2008) causes depletion of extracellular sodium and calcium proven to be a primary cause of death from Cu exposure in freshwater organisms if they are not able to recover (Grosell et al. 2002).

In light of previous studies and the observations made herein, toxicity and characterisation data obtained from the pilot study, it was hypothesized that, given the poor dissolution and high agglomeration of the CuO NMs, acute toxicity of the pristine CuO NMs was mostly due to ingestion of the NMs present at the bottom of the exposure vessel and subsequently death was caused either by impairment of the gut functionality (Croteau et al. 2011a) or internal dissolution and/or transformation of the CuO NMs in the acidic environment of digestive tract or digestive gland (Baun et al. 2008a, Golobič et al. 2012), where it has been demonstrated they can accumulate (Dummee et al. 2015). Thus, to limit the dietary exposure of Cu from the agglomerate NMs on the vessel's bottom, SbyD CuO NMs were manufactured, coating pristine CuO NMs with coating agents that would improve their suspension stability and thus reduce agglomeration and precipitation. However, the chemical components in the exposure medium plays a pivotal role and can significantly modify NMs fate and toxicity, as demonstrated in previous studies with CuO NMs (Blinova et al. 2010). Hence, in order to correctly interpret toxicity data, the SbyD CuO NMs were synthesised using two different dispersion agents (PBS and Milli-Q water) and accurately investigated in

either exposure medium or just Milli-Q water, to link the physicochemical characterisation to toxicological results.

The initial hypothesis was confirmed by characterisation and toxicity data gathered from experiments using CuO-ASC(PO_4^{-3}) NMs. In fact, for this specific NM, although the amount of dissolved Cu increased compared with the pristine NMs (Tab. 2-1), a smaller HDD was measured, and exposures led to a significantly decreased toxicity ($LC20_{96h}$ 1471 μgL^{-1}) compared with the pristine CuO NMs (Fig. 2-5).

However, once the collective data gathered from all the acute experiments with the SbyD NMs were examined, it was clear that other mechanisms of toxicity needed to be considered and that the interaction between the dispersants (Milli-Q water and PBS), coating agents and organisms might have triggered different specific effects for each NM.

To begin with, it was evident that the presence of PBS in the stock of SbyD suspensions had a strong influence on the toxicity of the CuO NMs. Indeed, in contrast to CuO-ASC(PO_4^{-3}), acute lethal toxicity of pristine CuO(PO_4^{-3}) NMs and CuO-PVP(PO_4^{-3}) to juveniles of *L. stagnalis* at 96h was around 10-fold of magnitude higher than to pristine CuO NMs dispersed in just Milli-Q water (Tab. 2-1). The use of a phosphate buffer that interacts strongly with a metal cation such Cu^{2+} may affect the bioavailability of the metal itself (Hughes and Poole 1991). For instance, Zevenhuizen et al. (1979) investigated the change in sensitivity to copper of bacteria in copper-buffered complex medium. Authors showed that for metal concentrations not exceeding to 2 mgL^{-1} Cu, soluble complex were formed at pH 7 with 0.5% phosphate buffer; at higher concentrations of Cu^{2+}, instead, insoluble copper-phosphate complexes precipitated, increasing thus the tolerance of the bacteria to Cu due to the limit amount of free Cu^{2+}. Furthermore, more recently, Conway et al. (2015) demonstrated the instability of nano-Cu in different natural media. At low pH an increased dissolution of the CuO NMs and subsequent formation of insoluble $Cu_3(PO_4)_2$ precipitate was recorded.

Similar phenomena might have happen in these experiments, where snails may have used the precipitates as a source of food and subsequently became exposed either by abstracting the metal ion directly or by producing ligands that complex and solubilize the metal ion (Hughes and Poole 1991).

This hypothesis seems to be confirmed in the exposure of snails to CuO-PVP(H_2O), where in absence of PBS an increase on colloidal stability was recorded in OECD 203 medium (HDD CuO-PVP(H_2O) = 1159 nm; CuO-PVP(PO_4^{-3}) = 2078 nm), resulting in $LC50_{96h}$ of 1007 μgL^{-1} around 6 times lower than that in PBS (Tab. 2-1).

Interestingly, the same relationship between reducing HDD and toxicity was not recorded for the two pristine CuO NMs suspended in the different dispersants (HDD CuO(H_2O) NMs = 2558 nm; CuO(PO_4^{-3}) NMs = 2364 nm). In fact, the HDD in OECD 203 did not change between the two different dispersants, nevertheless snails' mortality was 9-fold higher in experiments with CuO(PO_4^{-3}) NMs than with CuO NMs in just Milli-Q water.

This suggests that in the case of SbyD NMs functionalised with PVP, other factors are likely to have played a role on the toxicity of the NMs. ζ-potential represents another important parameter directly affecting colloidal stability and interaction of NMs with the surrounding medium and it depends on the composition and pH of the environment in which the NMs are dispersed (Ortelli et al. 2017). All the NMs investigated in this study, presented a ζ-potential indicating suspension instability, and overall no big differences were found between the samples functionalised in the different dispersant once diluted in OECD 203, except for the samples of CuO-PVP(H_2O) (Tab. 2-1). In this case, interestingly, although a decrease in HDD was measured compared with the counterpart in PBS (as expected), an increase, and change in charge of ζ-potential to +1.6 mV, was determined in contrast with the ζ-potential of -6.68 mV of the CuO PVP in PBS (Tab. 2-1).

Close to the isoelectric point, NMs have no charge resulting in a substantial weakening in the repulsive forces between NMs, which implies that each collision between primary particles and agglomerates causes particle adherence, leading to a high HDD NM suspension. Sousa and Teixeira (2013) investigating the agglomeration and kinetics of CuO NMs, demonstrated that at the isoelectric point, which for their NMs was found to be at pH 10, CuO NMs (mean particle size 50nm) presented their highest hydrodynamic diameter (1581 ± 137 nm), which resulted in larger agglomerates that settled rapidly owing to gravitational forces. Results from this research study are in contrast to Sousa and Teixeira (2013), however different medium, NMs particle size, pH and, most importantly, coatings were used; thus findings might not fully be comparable. Nevertheless, it can be suggested that steric stabilisation facilitate by PVP, in this case, is likely to have played a main role in the reduction of the toxicity compared to the counterpart in PBS.

In the case of the SbyD materials functionalised with ASC, the amount of adsorbed coating agent on the core material, and the amount of free ASC still in suspension might have influenced the toxicity of the two different materials, CuO-ASC(PO_4^{-3}) and CuO-ASC(H_2O) NMs. Indeed, thermographic analysis showed that in CuO-ASC(PO_4^{-3}) only

31% of the ASC was adsorbed on the core material compared to 61% for the CuO-ASC(H_2O), thus at same level of Cu concentrations the amount of free ASC in suspension was different. Ascorbate can serve either as antioxidant, in free radical-mediated oxidative processes, or pro-oxidant agent, reducing redox-active metals such as Cu^{2+}(Drouin et al. 1996), however it is unclear whether or not ascorbate directly reacts with CuO NMs (Buettner and Jurkiewicz 1996).

The crossover of ascorbate from antioxidant to pro-oxidant, depends from on its concentration, pH and the presence and concentration of catalytic metals such as Cu (Buettner and Jurkiewicz 1996). This threshold concentration is relative to metal species and exposure environment; however, the general principle that at relative low concentrations, ascorbate is likely to be a pro-oxidant, and at high concentrations, it will tend to be an antioxidant, is generally accepted (Buettner and Jurkiewicz 1996, Fukui et al. 2017, Zhao et al. 2017).

This principle can be applied here, and can help elucidate the results obtained in this research study. Indeed, as previously described, exposure to CuO-ASC(PO_4^{-3}) NMs resulted in a much lower acute effect compared with the pristine CuO NMs. Thermographic analysis showed that for CuO-ASC(PO_4^{-3}) 68% of the ASC was free in solution, thus ASC acting as antioxidant might have mitigated the toxicity of the core material resulting in lower snail. In contrast, in experiments with CuO-ASC(H_2O), where ASC free in solution was only 39%, the ASC might have served as pro-oxidant promoting the redox cycling of Cu between Cu(I) and Cu(II) which results in the production of ROS through Fenton and Harber–Weiss reactions (Zhao et al. 2017). This might have led to an additional toxicity effect of the pristine CuO NMs inducing a ≈ 10 time's fold mortality with an estimated $LC50_{96h}$ of 245 μgL^{-1} Cu (Tab 2-1).

Interestingly, once in OECD 203 medium, agglomerate size does not seem to be related with sedimentation rate in any of the NMs tested, suggesting that coatings and dissolution were likely to be stronger predictors of the sedimentation rate. It needs to be highlighted, that in this study all the characterisation experiments were performed using samples at much higher concentration of nominal Cu (50 mgL^{-1}) compared with those in the actual experiments. Furthermore, the suspension methods used by the two partners conducting the characterisation studies were different from each other and different from the methods used in the ecotoxicity studies.

Finally, samples for the characterisation experiments were collected in the absence of the exposed organisms, which could have led to the overlooking of the NMs' biotransformation carried out by the snails. For instance, Baumann et al.(2014) found

that in the presence of daphnids, stock of iron NMs coated with ASC (250mgL^{-1} Fe), reached the same colloidal instability visible by eye as in the one-year-aged stocks, which the authors attributed to turbulences induced by the swimming and filtering movements of the daphnids which might have accelerated the oxidization of ASC (Baumann et al. 2014).

Thus, although *L. stagnalis* exhibit a much slower motion compared to neonates of *Daphnia magna*, their grazing and swimming would have caused re-suspension and potentially increased bioavailability of the NM (Miseljic and Olsen 2014). Furthermore, the production of organic matter of by the snails could have interfered with the NMs behaviour.

2.5 Conclusions

In conclusion, the surface functionalization of NMs as well as the NMs' dispersant medium had a clear influence on their resulting toxicity for the juveniles of *L. stagnalis*. Indeed, on this study the importance of considering the exposure conditions when studying the fate and effects of engineered NMs, including SbyD, was highlighted.

Findings showed that PBS was not always a satisfactory medium to prepare NMs suspensions. This was also previously indicated by Sager et al (2007). The formation of agglomerates when using PBS and their interaction with metal NM in suspension likely increases the bioavailability of the NM to benthic organism such as *L. stagnalis*. Indeed, in the case of SbyD CuO NMs functionalised with PVP, SbyD CuO-PVP(PO_4^{3-}) NMs, the presence of PBS increased strongly the toxicity of pristine CuO NMs of around 10-fold. In contrast, functionalisation of pristine CuO NMs with ASC in PBS, SbyD CuO-ASC(PO_4^{3-}) NMs, resulted in a marked decrease in toxicity ($LC20_{96h}$ 1471) due to the antioxidant capability of ASC.

However, several knowledge gaps need to be filled to gain a comprehensive understanding of CuO NMs ecotoxicity and their corresponding mechanisms. Firstly, the characterisation of the NMs should be integrated with samples taken during and at the end of the exposure with organisms, so that the importance of biotransformation can be included. Furthermore, long term exposure at low exposure levels, reflecting real environment exposure conditions, are needed to fully understand the mechanisms of toxicity of the pristine NMs and the influence of the coating agents in enhancing or mitigating toxicity of the former.

Chapter 3 Reprotoxicity and growth effects of CuO NMs on young adults of the snail, *L. stagnalis*

3.1 Introduction

Copper oxide nanomaterials (CuO NMs) are one of the most commercially used NMs in industrial and consumer products. Due to their widespread use, there is a particularly high risk of a runoff of these materials in the aquatic environment, where due to their small size and physico-chemical proprieties may have detrimental effects to diverse organisms (Nowack et al. 2012b, Keller and Lazareva 2014).

As previously described in Chapter 2, the acute toxicity of CuO NMs to aquatic organisms has been assessed in a variety of studies (Blinova et al. 2010, Gomes et al. 2011, Gunawan et al. 2011, Gomes et al. 2012, Bondarenko et al. 2013, Buffet et al. 2013). Results vary considerably among species, CuO NMs physico-chemical proprieties, media and exposure conditions; which therefore result in different levels of toxicity due to the different interactions between the NMs, media and the different organisms (Wu et al. 2017).

Although acute toxicity studies provide useful information on contaminant hazards, studies investigating the direct and indirect toxicity effects of NMs, due to long-term exposure in aquatic ecosystems, can provide a more wide-ranging estimation of the potential ecotoxicity of a given NM (Adam et al. 2015, Wu et al. 2017). Unfortunately for time and financial reasons, compared to acute studies, only few studies have evaluated, so far, the chronic toxicity of CuO NMs to aquatic organisms (Liu et al. 2014, Ramskov et al. 2014, Rossetto et al. 2014, Zhang et al. 2014, Adam et al. 2015, Ates et al. 2015, Nations et al. 2015, Wang et al. 2015, Ma et al. 2017, Wu et al. 2017).

In ecotoxicological studies invertebrates have been extensively used as model test species, as they are abundant, distributed worldwide and good biomonitor to assess the risk of chemicals including NMs (*e.g.* Brix et al. 2011, Pradhan et al. 2012, Hanna et al. 2013, Munley et al. 2013). In particular, aquatic gastropods, in addition to their importance in freshwater systems, have demonstrated high sensitivity to sublethal levels of metals, allowing an early detection of their potential impact within an ecosystem (Byzitter et al. 2012).

Chronic toxicity of CuO NMs to gastropods, to date, has been evaluated only by few studies focussed on sediment-associated freshwater snails (Ramskov et al. 2014, Ma et al. 2017, Pang et al. 2012). Ma et al. (2017) assessed the comparative toxicity of sediment-associated CuO NMs, CuO microparticles (10 μm) (MPs) and ionic Cu, to evaluate chronic bioaccumulation and oxidative stress biomarker responses in the adult of the deposit-feeding freshwater snail, *Bellamya aeruginosa*. Results indicated that *B.*

aeruginosa accumulated Cu, after 28 days of exposure, from sediment-associated CuO NMs by direct ingestion mainly in the hepatopancreas and gonad. Furthermore, only exposure to CuO NMs and ionic Cu caused oxidative stress (but not to CuO MPs), attributing thus the toxicity of CuO NMs to specific nanoparticulate effects (Ma et al. 2017).

Accordingly, Pang et al. (2012) and Ramskov et al. (2014) demonstrated that spiked sediment with CuO NMs or ionic Cu caused bioaccumulation and negative effects on life-history traits, such as reproduction, growth and feeding rate, of on deposit-feeding snail, *Potamopyrgus antipodarum*. Ramskov et al. (2014) exposed for nine weeks the snails to either aqueous Cu or CuO NMs (240 mg Cu g^{-1} dry weight) of different shapes (rods, spheres, or platelets). Results indicated no clear correlation between accumulation and adverse sublethal effects, indicating that further studies where needed to better understand the uptake mechanisms and internal distribution pathways of the NMs (Ramskov et al. 2014).

Indeed, to date, of all the ecotoxicological studies of CuO NMs, no study has focused yet on the chronic effect on the reproduction and growth, key ecological aspects for the survival of any species (OECD 2016).

Chronic studies have already shown that for several metals (Co, Pb, Cd and Ni) *L. stagnalis* is either the most sensitive or second most sensitive species tested (Grosell et al. 2006, De Schamphelaere et al. 2008, Schlekat et al. 2010, Brix et al. 2011, Das and Khangarot 2011, Ducrot et al. 2014, Niyogi et al. 2014), thus it is important to compare these finding with their counterpart in the nano-form.

Indeed, this research study aimed to address this knowledge gap on the investigation of the long-term risks and potential mechanisms of toxicity of CuO NMs on the snails *L. stagnalis*. Based on the first steps for the approval of a OECD guidelines for the reproduction test for molluscs (OECD 2016) delineated in Ducrot et al. (2014), experiment were focussed on the detrimental effect on reproduction (reprotoxicity) of different CuO NMs (pristine and safer by design (SbyD) CuO NMs) and the ionic form of Cu. Furthermore, other life-history traits such as growth and feeding rate, which could have consequences for population dynamics, were investigated alongside the 30 days exposure.

Nanomaterials associated with a food source (feeding exposure) have been shown to elicit indirect or secondary toxicity effect on *L. stagnalis*. Indeed, Croteau et al. (2014b) observed that snails fed at a faster rate when offered diatoms mixed with ^{65}CuO NMs compared to ionic Cu-laden diatoms resulting in a higher uptake of the former.

However, increasing exposure concentration did not translate into an inhibition of feeding, which remained relatively constant across the high dietary concentrations (Croteau et al. 2014b). In contrast, when in two different studies, *L. stagnalis* were exposed to zinc oxide (ZnO) NMs (Croteau et al. 2011a) and Ag NMs (Croteau et al. 2011b) a decrease in feeding rate up to 4-orders of magnitude compared with their ionic counterpart was shown, which resulted to gut and food ingestion impairment. In addition, Croteau et al. (2011b) observed an arrest in growth of *L. stagnalis* exposed to a diet spiked with Ag NMs coated with citrate due to a decrease in feeding rate and damaged digestion.

Reduction on food consumption along with disruption of gut functionality can produce adverse effects that can influence higher level processes like growth and reproduction, and ultimately alter populations' distributions and dynamics (Croteau et al. 2011b). Indeed, Ter Maat et al. (2007) highlighted the correlation between growth and reproduction output, when studying the influence of food availability and day length on *L. stagnalis*. These authors observed that, after 2 months, the size of snails kept under 16:8 h light condition (as was done for the snails' object of this research study) depended on food availability. Snails fed the lowest quantities grew the least causing a secondary effect on their reproduction activity, which declined with declining food availability. In contrast, when snails reared under 12:12 h light condition, were not affected by food availability and grew increasingly along the 2 months of exposure.

Chronic exposure to CuO NMs, as previously reported, has been reported to affect negatively the reproduction behaviour of other snails species (Pang et al. 2012, Ramskov et al. 2014), crustacean (Wu et al. 2017), and fish (Amde et al. 2017); however, to date, no study has evaluated the effect of CuO NMs, and NMs in general, on the reproduction of *L. stagnalis*.

This study aimed to develop a partial life-cycle test to investigate the effects of different CuO NMs on the reproduction of the freshwater gastropod *L. stagnalis*, which has been identified as model test species to assess reprotoxic effects of chemicals (OECD 2016), adapted from the protocol developed by Ducrot et al. (2014). Furthermore, endpoints such as growth and feeding rate were also evaluated along with the reproduction parameters.

3.2 Materials and methods

3.2.1 Experimental design

In this study, young adult of *L. stagnalis* (22 ± 2 mm) were exposed in a semi-static experiment to Cu as CuO NMs and $CuSO_4$ for 30 days. To allow results homogeneity, shell size length was measured using a digital calliper at the start of the experiment. Four replicates, of five snails each, were exposed to each concentration and controls in a 1 L glass beaker. Snails were allowed to acclimatise in the new vessel and exposure started only when reproduction occurred again. At the end of the acclimation period, test vessels were filled with 950 ml of clean test medium and total body mass (wet weight) per replicate was determined, blotting dry snails on paper towels (Ducrot et al. 2014). All the endpoints were determined at the start, end (30 days) and after 3, 9, 12, 15, 21, 24 and 27 days of exposure.

All exposure concentrations were based on mass of copper added; dosing was accomplished by adding the desired aliquot from the stocks into the exposure vessels. The concentration ranges for pristine CuO NMs and $CuSO_4$ were selected based on their potential to induce lethal effects, allowing the plot of logistic curves from which LC50 values were estimated. Concentration ranges (0-200 μgL^{-1} Cu) for the SbyD CuO NMs were chosen based on the chronic lethal toxicity of pristine CuO NMs.

Snails were fed *ad libitum* and a 100% medium renewal was performed every 3 days to maintain exposure concentrations over the experiment duration and water quality. At the same time, clutches/eggs were removed from the glass walls, using a sharp-edged metallic spoon and fecundity endpoints (cumulative number of clutches/eggs per individual per day) per replicate were determined (Ducrot et al. 2014). Mortality was also recorded at each time point; individuals may have reproduced before dying and therefore contribute as well to the reproduction output recorded at the specific time point. Thus, to avoid any bias in the statistical analyses, in computing the fecundity endpoints values, when a snail was alive at time *t* but counted as dead at time (t+1), it was then assumed to be actually dead at $((t+1)+t)/2$ (Charles et al. 2016).

At the end of the experiment at day 30, feeding rate was determined following Brix et al (2012). Briefly, the relationship between lettuce wet weight (prior to being placed in the test vessel) and area was determined. Individual pieces of lettuce were photographed before and after snails were allowed to feed for 24 h. Images where then digitally analysing the image using the free area analysis software Image J (Rasband 2011), and differences in area before and after were then used to estimate the mass of lettuce

consumed by individual snails and then normalized for differences in snail mass (weight of the food consumed per day (mg)/wet weight of snail (mg)).

3.2.2 Test chemicals and NMs characterisation

Nanomaterials used in this experiment and their characterisation have been previously described in Chapter2, section 2.2.1.

In this instance, further analyses were performed by the project partner, ISTEC-CNR, to assist the interpretation of the results obtained from the chronic tests. Characterisation to determine solubility, ζ-potential and HDD of all the CuO NMs tested, were conducted on samples simulating testing experimental condition. In brief, initially all the stock suspensions of the SbyD CuO NMs, functionalised either in Milli-Q water or in phosphate buffer, were diluted in OECD 203 medium from 10 gL^{-1} to experimental test concentration of 100 μgL^{-1}. Subsequently, from each sample 15 ml were placed into separated vials and positioned into a thermostatic bath (20 °C) for 3 days under magnetic stirring at 16/8h light condition, to mirror the amount of time between water renewal, temperature and light during the 30 days of chronic experiment.

3.2.3 Data analyses

SigmaPlot© version 13.0 (Systat Software, Inc.) was used to perform all the statistical analyses. All data were checked for normality of distribution Shapiro–Wilk test and for homogeneity of variances using Levene or Mauchly's tests Correlations were assessed by computing Pearson's product moment statistic. Logistic regression analyses followed by repeated measure analyses of variance (RMANOVA) were used to establish significant differences between time points within the same exposure scenario. Differences between different experiments were analysed using analysis of variance (ANOVA) with Tukey's (all pairwise) or Dunnett's (*vs* control) post-hoc analysis. In case data did not pass the Shapiro–Wilk normality and the Levene's homogeneity of variance tests, the non-parametric Kruskal–Wallis test (one-way ANOVA on ranks) was used. All differences were considered statistically significant at $p < 0.05$. Lethal and effective concentration values (L/EC50) affecting 50% of the population were calculated using a non-linear allosteric decay function in a spreadsheet built over Microsoft Excel (ToxCalcMix) (Barata et al. 2006).

3.3 Results

3.3.1 Characterisation of CuO NMs

Results obtained from secondary characterisation of the different CuO NMs has been already described in detail in Chapter 1, section 1.2.3 and Chapter 2, section 2.3.1; here a table (Tab. 3-1) summarising in brief the results is presented.

Table 3-1 Summary of characterisation analyses of CuO NMs in OECD 203 medium (data are means ± SEM).

	d_{DLS} (nm)	$Cu_{dissolved}/CuO_{total}$ (%)	*ζ-pot*$_{ELS}$ (mV)	
		t = 24h, T = 20°C		pH
Pristine CuO NMs (H_2O)	2558 ± 472	< 0.02	-4.36 ± 0.57	7
CuO-PVP(H_2O) NMs	1159 ± 256	0.1	+1.6 ± 0.3	8.1
CuO-ASC(H_2O) NMs	1293 ± 278	0.3	−8.1 ± 0.4	8.1
CuO(PO_4^{3-}) NMs	2364 ± 282	0.093	-3.37 ± 0.1	7.12
CuO-PVP(PO_4^{3-}) NMs	2098 ± 545	0.071	-6.68 ± 0.1	7.25
CuO-ASC(PO_4^{3-}) NMs	1719 ± 157	0.896	-9.52 ± 0.2	6.72

Results obtained after the simulation tests, diluting the stock suspension at the working concentration of 100 μgL^{-1} and subjecting the sample to three days of 16/8h light cycle at 20 °C, are reported in Table 3-2.

Table 3-2 HDD and zeta potential and dissolution data of pristine and SbyD CuO NMs suspensions diluted 100 μgL^{-1} in OECD 203 medium (error bars are SEM).

	d_{DLS} (nm)	$Cu_{dissolved}/CuO_{total}$ (%)	ζ-potELS (mV)
Pristine CuO NMs (H_2O)	417.3 ± 15.0	60.2	-7.95 ± 1.0
CuO-PVP(H_2O) NMs	967.6 ± 447.7	68.2	-12.2 ± 1.8
CuO-ASC(H_2O) NMs	400.5 ± 34.8	74,2	-11.9 ± 0.7
CuO(PO_4^{3-}) NMs	838.5 ± 173.4	34.1	-6.77 ± 0.7
CuO-PVP(PO_4^{3-}) NMs	581.9 ± 32.1	29.4	-13.2 ± 1.2
CuO-ASC(PO_4^{3-}) NMs	ND	36,7	ND

Results for ζ-potential and HDD measurements confirmed the trend recorded in samples analysed at 10gL^{-1} Cu concentration (Tab. 3-1). Indeed, CuO NMs samples

functionalised in phosphate buffer showed a larger HDD compared with those in Milli-Q water, most likely due to the destabilization occurring in the presence of negative phosphate ions that reverse the positive zeta potential of CuO NMs when dispersed in ultrapure water (Tab. 1-3, Chapter 1). However, the difference between the samples was not so marked to be considered significant. Furthermore, all samples present a negative zeta potential inside the range of colloidal instability (± 30 mV), confirming preferential adsorption of inorganic anions, present in OECD203 medium, onto CuO NMs surface.
Results from dissolution data are instead very different. The dissolution was very high at such low concentration, and over 3 days, reaching values around 70% dissolution for the samples functionalised in Milli-Q water and around 30 % for those in phosphate buffer. Overall, samples from CuO NMs functionalised in phosphate buffer showed a lower dissolution than those in Milli-Q, probably due to solvation of the Cu^{2+} ions and subsequent precipitation of solid copper phosphate, which solubility is lower.

3.3.2 Chronic toxicity of aqueous Cu and CuO NMs

3.3.2.1 Chronic lethality of aqueous Cu and CuO NMs on young adults

Initial studies were performed investigating the chronic lethality at 30 days of pristine CuO NMs (H_2O) and $CuSO_4$, as ionic Cu control, to young adults (22±2 mm) of the freshwater gastropod *L. stagnalis*. Nominal exposure concentrations were chosen ($CuSO_4$: 0-80 μgL^{-1} Cu; pristine CuO NMs: 0-1500 μgL^{-1} Cu) for their potential to induce lethal effect, to establish a sublethal concentration range, which would inhibit vital functions without causing death, for further tests using the SbyD CuO NMs. Overall, results showed a 7-fold higher chronic toxicity of the ionic form Cu as $CuSO_4$ compared to the pristine CuO NMs.
Snails exposed to 80 and 60 μgL^{-1} Cu of $CuSO_4$ resulted in 80% and 45% mortality after 30 days exposure, whereas, in the same period, in the control and in the 20 and 40 μgL^{-1} Cu treatments mortality did not exceed 5 % (Fig. 3-1). Nevertheless, despite the high survival rate, snails exposed to 40 μgL^{-1} Cu exhibited a clear change from the normal locomotion behaviour (not quantified); snails appeared to prefer the upside-down gliding (Aono et al. 2008) in clusters instead of the normal attachment to the exposure vessel's walls. Findings indicated that snails were affected by the exposure to ionic Cu only after 18 days and 15 days (30% mortality) at concentration of 60 μgL^{-1} Cu and 80 μgL^{-1} Cu, respectively. Snails reached 55% mortality in the highest concentration after 18 days of exposure. LC50 value estimated at 30 days was 63.46 (± 2.26 SE) μgL^{-1} Cu

(Fig. 3-1). The effect of ionic Cu was significant in the induction of mortality over the 30 days of exposure (RMANOVA, $F_{(4,20)} = 2.87$; $p = 0.041$), however post hoc analysed (Tukey test) found not significant difference between days (Fig. 3-1).

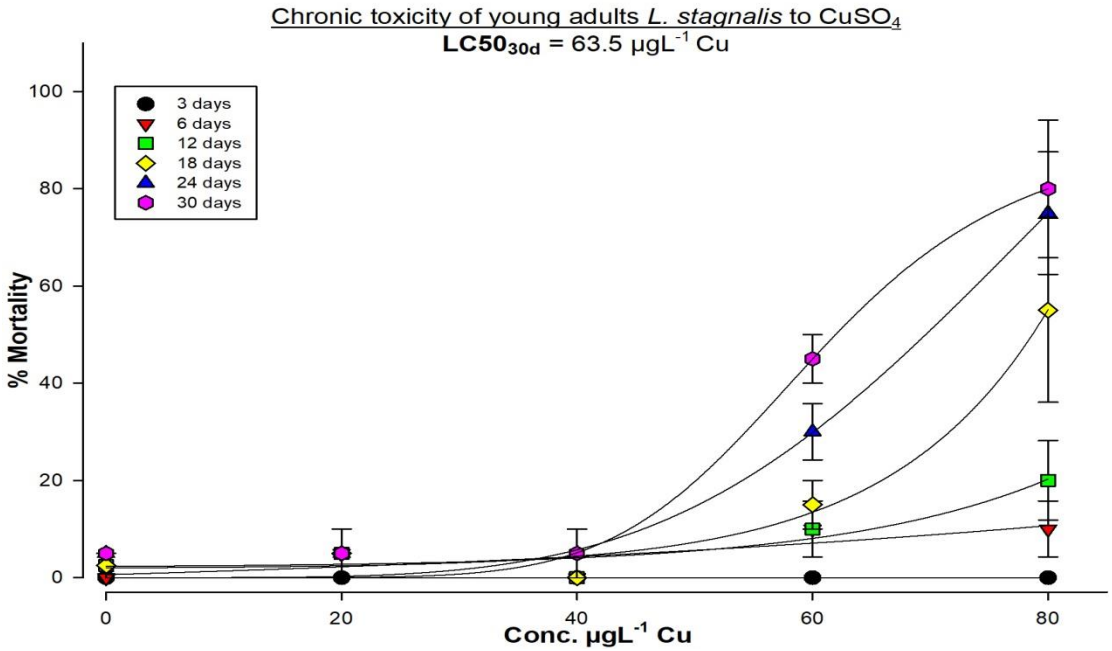

Figure 3-1 Percentage mortality (%) of young adults of *Lymnaea stagnalis* (≈ 22 ± 2 mm) exposed to increasing concentrations of Cu as $CuSO_4*5H_2O$ at 20 ºC for 30 days. Solid lines stand for the fitted 4-parameters logistic (4PL) model (n = 4, error bars are SEM).

Clearer concentration-response and time dependant relationships were, in contrast, observed in snails exposed for 30 days to pristine CuO NMs (Fig. 3-2). At the two highest concentrations tested (1000 and 1500 µgL^{-1} Cu) over 50 % of mortality was recorded after 6 days of exposure, reaching 100% mortality at 12 days of exposure. At 800 µgL^{-1} Cu, 100% mortality was observed at the end of the experiment. LC50 calculated at 30 days for snails exposed to pristine CuO NMs was 492.2 (± 18.42 SE) µgL^{-1} Cu. Data failed the normality Shapiro-Wilk test ($p > 0.05$), thus a one-way repeated measures ANOVA on ranks (Friedman Test) was performed, which indicated that there was a statistically significant difference in lethal toxicity between time points, $\chi^2(5) = 53.49$, $p < 0.001$. Post hoc analysis conducted with a Tukey test indicated significant differences between the median mortality at 30, 24 days vs 3 and 6 days of exposure. In addition, median mortality at 12 days was significant different from the 30 days of exposure.

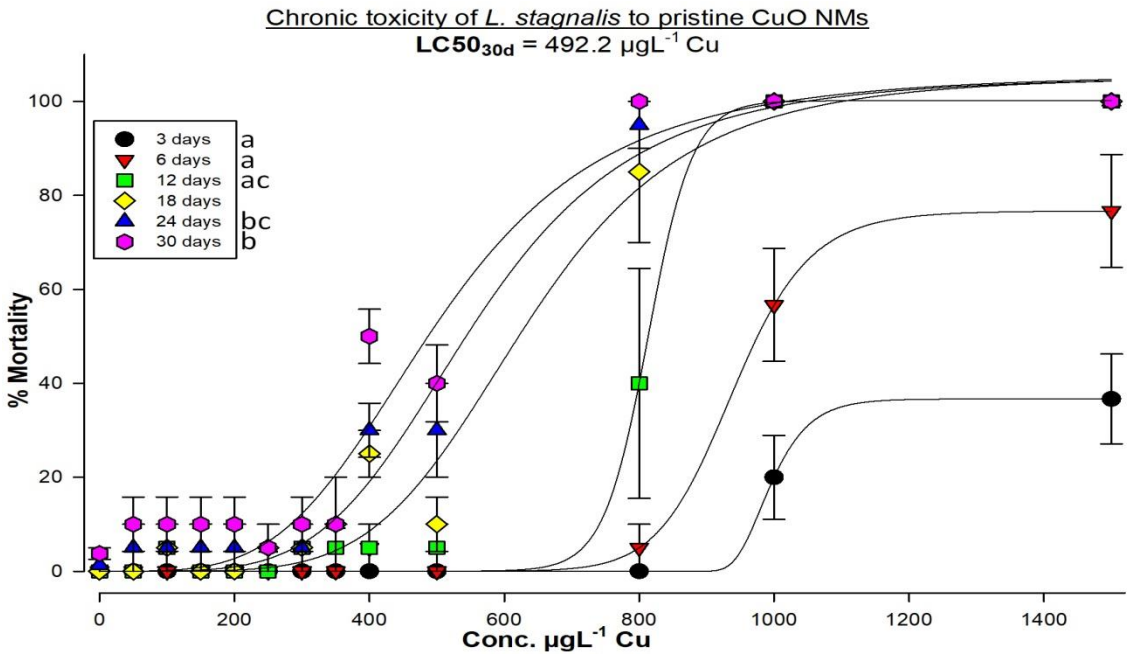

Figure 3-2 Percentage mortality (%) of young adults of *Lymnaea stagnalis* (≈ 22 ± 2 mm) exposed to increasing concentrations of Cu as pristine CuO NMs at 20 ºC for 30 days. Solid lines stand for the fitted 4PL model; different letters indicate significant differences $p < 0.05$ (n = 4, error bars are SEM).

Experiments using the SbyD CuO NMs were conducted with the range of concentrations (0-200 µgL^{-1} Cu) (Fig. 3-3). Results showed that at 30 days mortality of snails exposed to the SbyD CuO NMs functionalised in Milli-Q water and CuO-ASC(PO_4^{3-}) NMs did not exceed 25%. Mortality induced, at the same concentration range, by the pristine CuO NMs, is represented in Fig. 3-3 with red stars. In contrast, snails exposed to CuO(PO_4^{3-}) NMs at 30 days showed a slightly higher mortality, 35%. Thus, at 30 days the derived LC30 value was 186.27 (± 33.62 SE) µgL^{-1} Cu. In contrast, and in agreement with the results obtained from the acute lethal toxicity studies (see Chapter 2, section 2.3.2), snails exposed to CuO-PVP(PO_4^{3-}) NMs exhibited 80% of mortality after 30 days of exposure (Fig. 3-3), resulting in a LC50 value of 160.05 (± 15.65 SE) µgL^{-1} Cu.

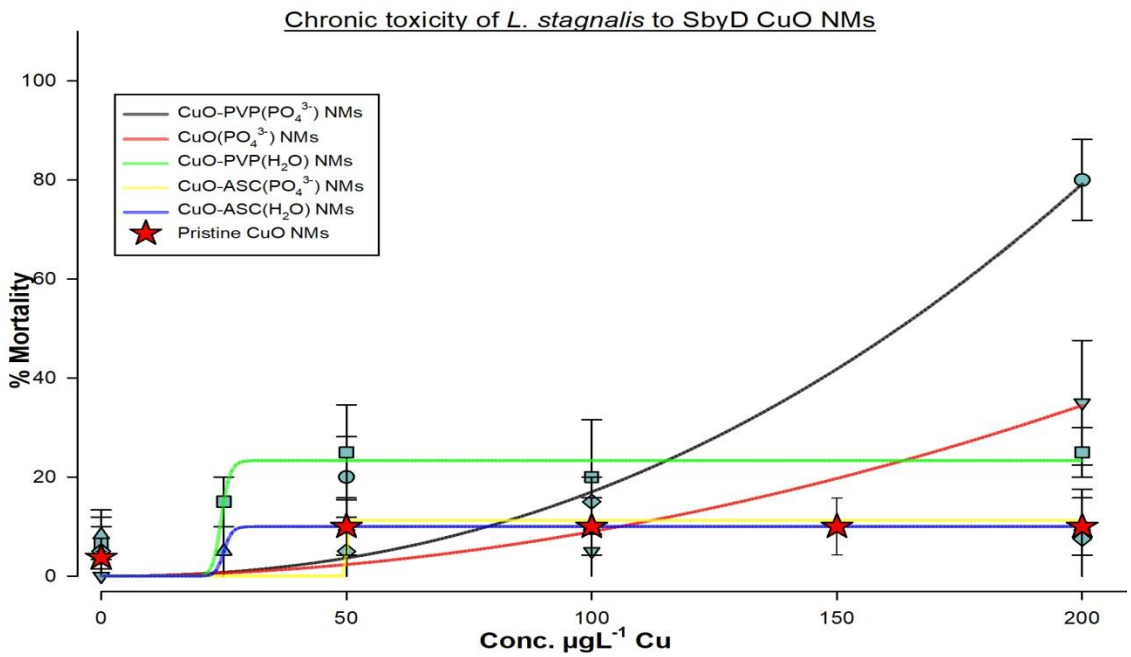

Figure 3-3 Percentage mortality (%) of young adults of *Lymnaea stagnalis* (≈ 22 ± 2 mm) exposed to increasing concentrations of all the tested SbyD CuO NMs at 20 ºC for 30 days. For comparison, mortality of snail, exposed to up to 200 µgL^{-1} Cu of pristine CuO NMs, is plotted represented by the red stars. Solid lines stand for the fitted 4PL model (n = 4, error bars are SEM).

3.3.2.2 Reprotoxicity of aqueous Cu and CuO NMs on young adults

Overall, the cumulative production of clutches and eggs per snail per day decreased in a concentration-dependent manner during the 30 days of exposure to aqueous or nano Cu. In accordance with the chronic lethality results, a greater toxicity of ionic copper compared with the pristine CuO NMs was induced for the endpoints investigated. At 30 days of exposure to $CuSO_4$, EC50 values estimated were 62.8 (± 3.8 SE) (Fig. 3-4) and 49.1 (± 3.6 SE) µgL^{-1} Cu (Fig. 3-5) for the cumulative number of clutches and eggs, respectively. A one-way ANOVA between different concentrations was conducted to compare the effect of $CuSO_4$ on the fecundity of the snails. A significant inhibition was revealed on the production of clutches and eggs per individual per day at the $p < 0.05$ level for the five treatments ($Fs_{(4, 19)} = 23.96$ (clutches)/ 50.77 (eggs), $p < 0.001$). Post hoc comparisons using the Dunnett's Method indicated that at 30 days the number of clutches and eggs produced in the 80 µgL^{-1} Cu and 60 µgL^{-1} Cu treatments were significantly less compared to the control ($p < 0.001$). At intermediate and lower concentrations tested (respectively 40 and 20 µgL^{-1} Cu), eggs production was also significantly different from the controls (Fig. 3-5). However, at the same concentrations there were no differences to the control in relation to the productions of clutches (Fig. 3-4).

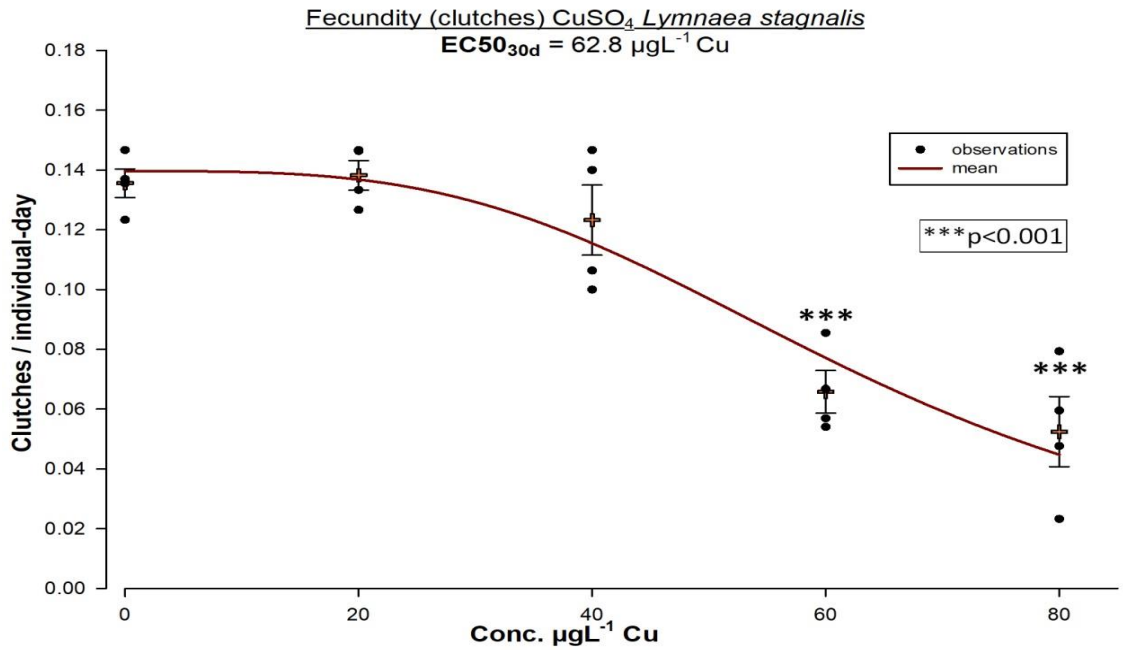

Figure 3-4 Cumulative number of clutches produced per snail per day exposed over 30 d to $CuSO_4$. Black dots stand for the observed values, brown plus symbols for the mean observed values, and brown line for the fitted model (n = 4; error bars are SEM). Asterisks indicate significant differences, $p < 0.05$, compared with the control.

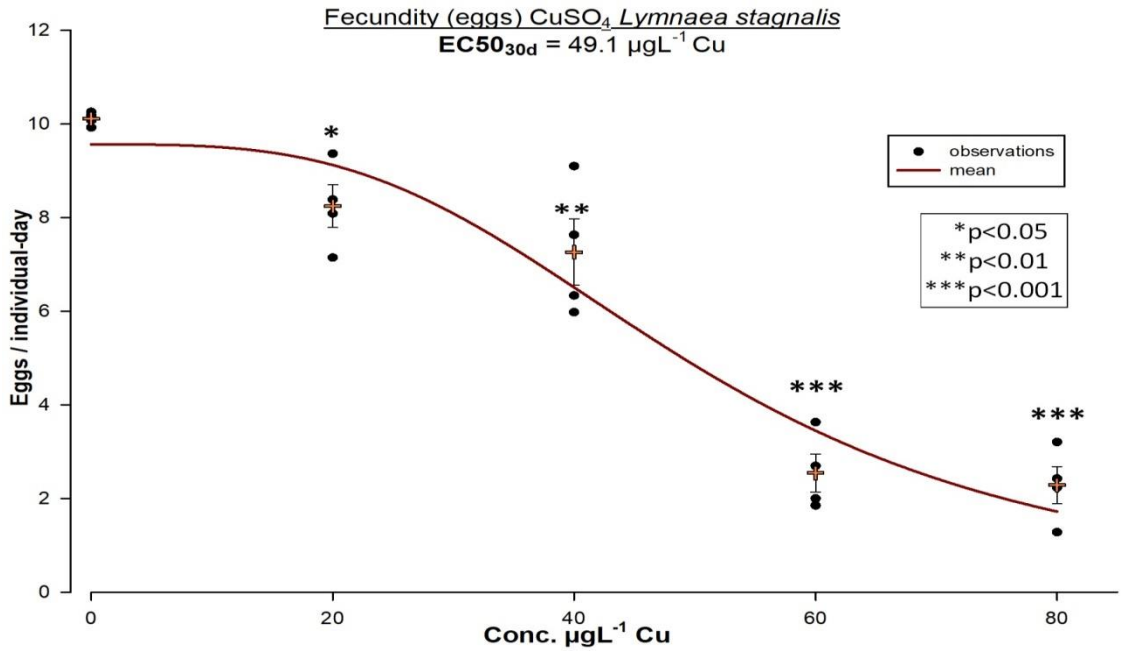

Figure 3-5 Cumulative number of eggs produced per snail per day exposed over 30 d to $CuSO_4$. Black dots stand for the observed values, brown plus symbols for the mean observed values, and brown line for the fitted model (n = 4; error bars are SEM). Asterisks indicate significant differences, $p < 0.05$, compared with the control.

Data collected from the reproduction test with pristine CuO NMs have been analysed only for exposure concentrations between 0-800 μgL^{-1} Cu since early mortality of all snails at exposure concentrations of 1000 and 1500 $\mu g\ L^{-1}$ Cu hampered the assessment of the reproductive effects. These data will thus not be discussed. EC50 estimates obtained from the fecundity data are 156.9 (± 22.14 SE) μgL^{-1} Cu (Fig. 3-6) and 131.9 (± 19.55 SE) μgL^{-1} Cu (Fig. 3-7) for cumulative numbers of clutches and eggs,

respectively. One-way ANOVA comparison revealed that the effect of Cu as pristine CuO NMs was significantly different across the different concentrations regarding reduction of fecundity, yielding F ratios of $Fs_{(10, 33)}$ = 12.73 (clutches)/ 12.65 (eggs); $p < 0.001$). At the concentration range, 200-800 µgL^{-1} Cu exposures resulted in a clear reduction in the numbers of clutches (Fig. 3-6) and eggs (Fig. 3-7) was revealed as compared with the unexposed control ($p < 0.001$). However, no significant difference to the control was observed for the snail exposed to 50 µgL^{-1} Cu of pristine CuO NMs.

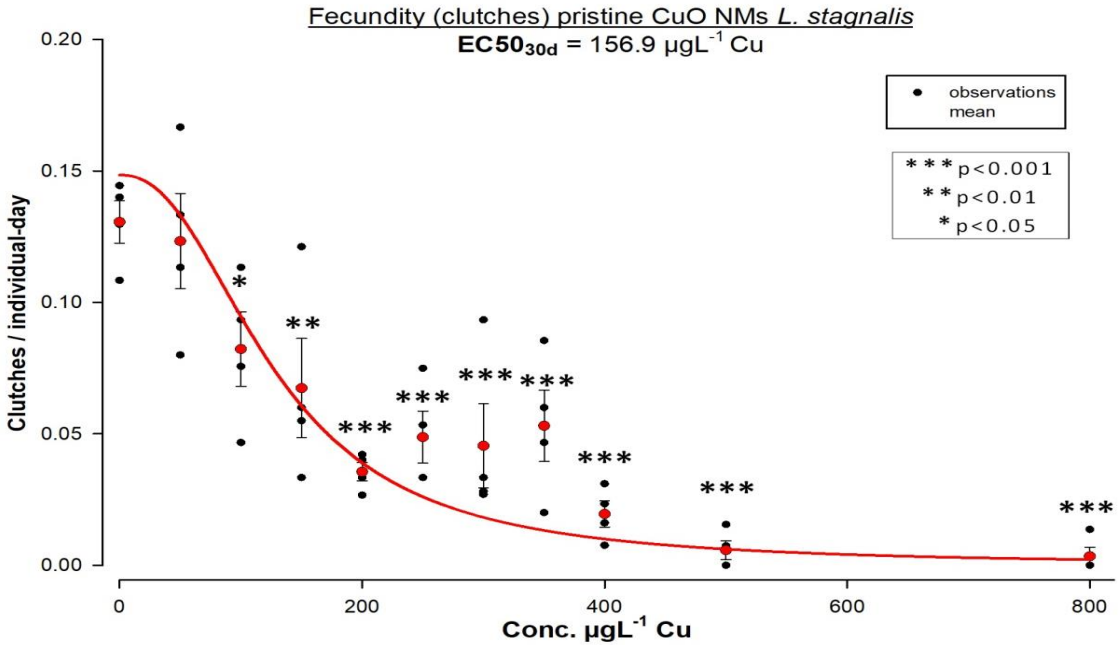

Figure 3-6 Cumulative number of clutches produced per individual snail per day over 30 d of exposure to pristine CuO NMs. Black dots stand for the observed values, red dots for the mean observed values, and red line for the fitted model (n = 4; error bars are SEM). Asterisks indicate significant differences, $p < 0.05$, compared with the control.

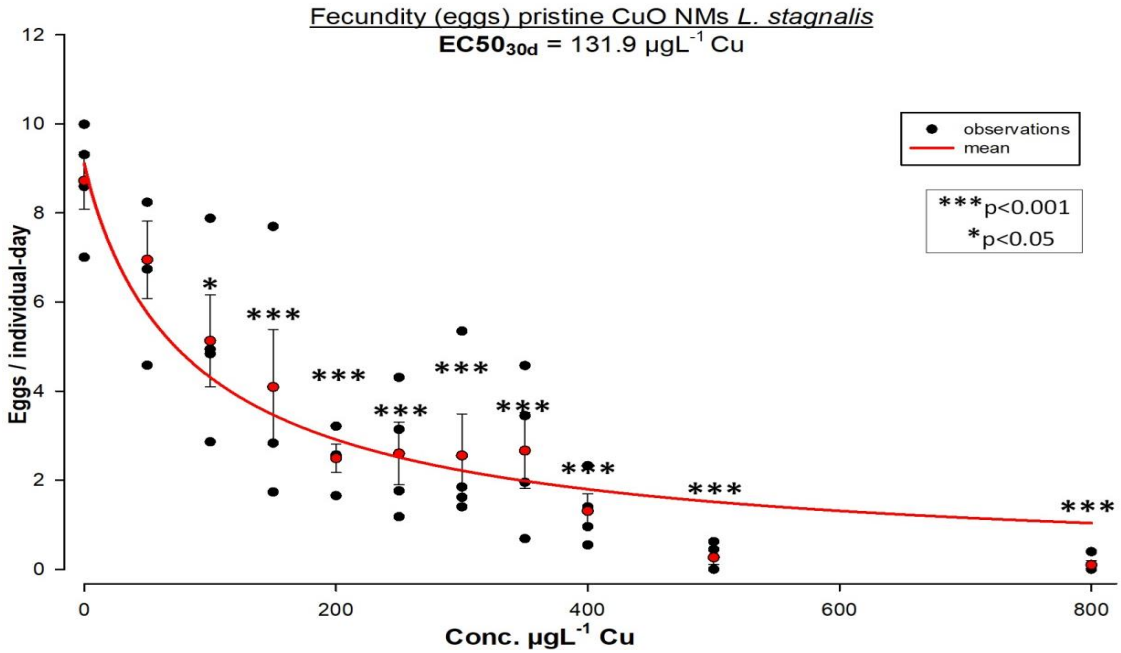

Figure 3-7 Cumulative number of eggs produced per individual snail per day over 30 d of exposure to pristine CuO NMs. Black dots stand for the observed values, red dots for the mean observed values, and red line for the fitted model (n = 4; error bars are SEM). Asterisks indicate significant differences, $p < 0.05$, compared with the control.

Findings from the experiments with the SbyD CuO NMs functionalised in Milli-Q water showed a higher toxicity, for both endpoints, of CuO-ASC(H_2O) NMs compared with CuO-PVP(H_2O) NMs. Nevertheless, toxicity was still lower than pristine CuO NMs and CuSO4. In particular, compared with the pristine CuO NMs, exposure to CuO-ASC(H_2O) NMs affected primarily, although not significantly the production of eggs (Fig. 3-7), and less the clutches (Fig. 3-8). Indeed, EC50 values at 30 days estimated following exposure to CuO-ASC(H_2O) NMs were 132.72 (± 31.54 SE) and 74.57 (± 16.76 SE) μgL^{-1} Cu for the cumulative numbers of clutches and eggs respectively. The latter value is half of the EC5030d value calculated for the effect of pristine CuO NMs on the eggs production at the same concentration range (0-200 μgL^{-1} Cu), represented by the dotted red line in Fig. 3-8 and estimated as 140.97 (± 24.82 SE) μgL^{-1} Cu. Interesting, results gathered from exposure to CuO-PVP(H_2O) NMs showed a significant inhibition in the fecundity of the snails compared with the control, even at the lower concentration tested of 25 μgL^{-1} Cu. However, with the increase in concentration up to 200 μgL^{-1} Cu no further decrease in the production of eggs and clutches was recorded, resulting in the impossibility of estimating of an ECx at 30 days (Fig. 3-7 and 3-8).

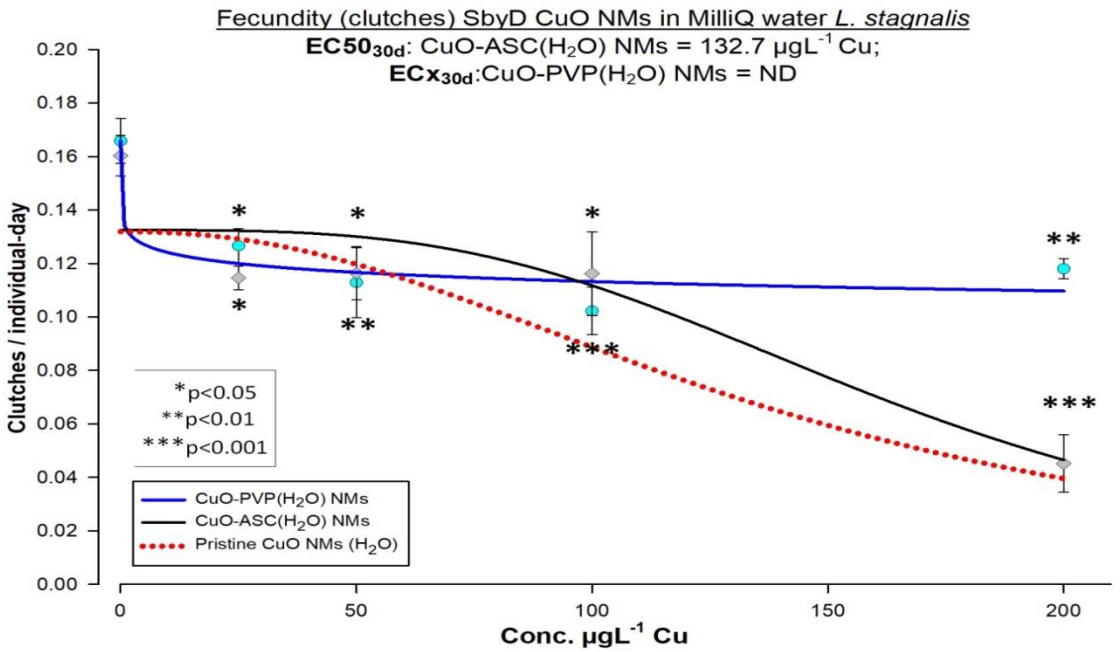

Figure 3-8 Cumulative number of clutches produced per individual-day over 30 d of exposure to SbyD CuO NMs functionalised in Milli-Q water. For comparison, the fitted model for snails exposed to up to 200 µgL^{-1} Cu of pristine CuO NMs is plotted, represented by the red dotted line (n = 4; error bars are SEM). Asterisks indicate significant differences compared with their respective control ($p < 0.05$).

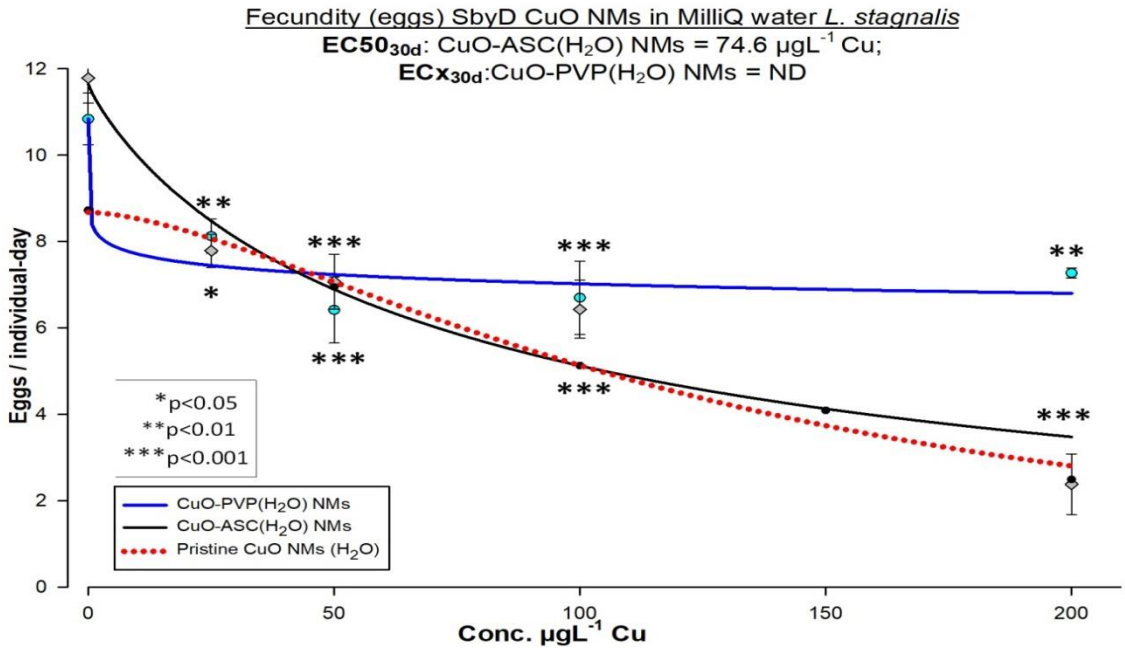

Figure 3-9 Cumulative number of eggs produced per individual-day over 30 d of exposure to SbyD CuO NMs functionalised in Milli-Q water. For comparison, fitted model for snails exposed to up to 200 µgL^{-1} Cu of pristine CuO NMs is plotted, represented by the red dotted line (n = 4; error bars are SEM). Asterisks indicate significant differences compared with their respective control ($p < 0.05$).

The progressive suppression in fecundity was also noticeable for the three different SbyD CuO NMs functionalised in PBS. The same trend regarding the stronger decline in the number of eggs compared to clutches was reported for snails exposed to CuO(PO_4^{3-}) NMs and CuO-ASC(PO_4^{3-}) NMs compared to with the pristine CuO NMs at the same concentration range (Fig. 3-10 and 3-11). EC50 values at 30 days estimated for exposure to CuO(PO_4^{3-}) were 116.36 (± 7.86 SE) µgL^{-1} Cu for clutches' production

and 78.21 (± 10.54 SE) µgL^{-1} Cu for the cumulative number of eggs. Similarly, EC50 values for exposure to CuO-ASC(PO_4^{3-}) NMs were 113.82 (± 21.56 SE) µgL^{-1} Cu and 73.379 (± 15.85 SE) µgL^{-1} Cu for the production of clutches and eggs respectively (Fig. 3-10 and 3-11).

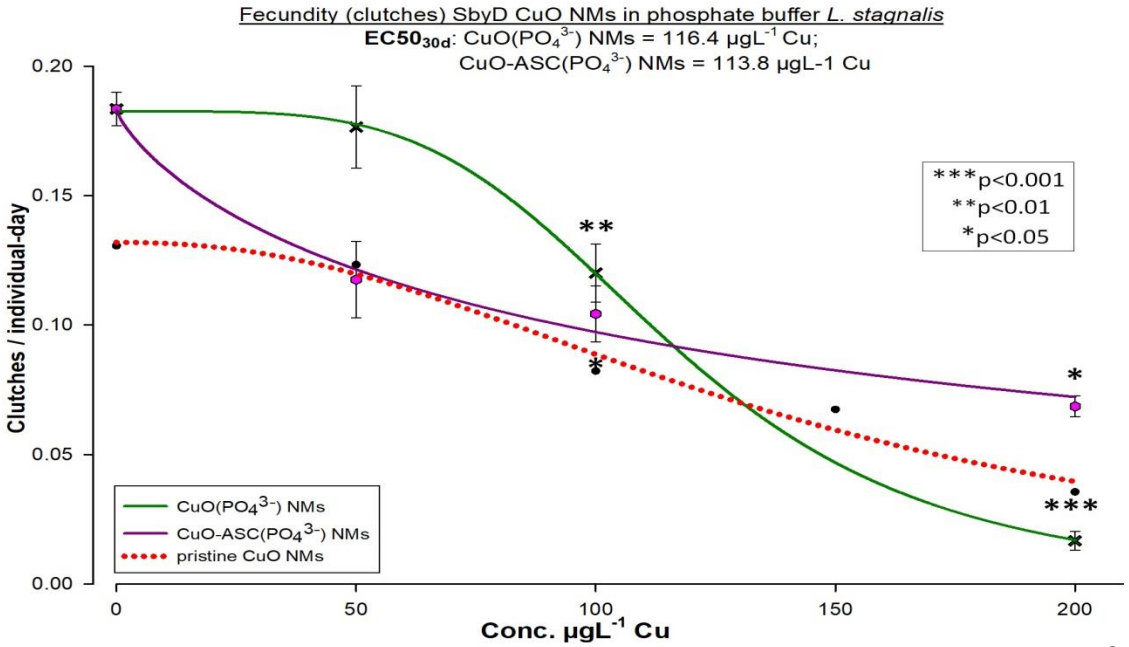

Figure 3-10 Cumulative number of eggs produced per individual-day over 30 d of exposure to CuO(PO_4^{3-}) NMs and CuO-ASC(PO_4^{3-}) NMs. For comparison, fitted model for snails exposed to up to 200 µgL^{-1} Cu of pristine CuO NMs is plotted, represented by the red dotted line (n = 4; error bars are SEM). Asterisks indicate significant differences, $p < 0.05$, compared with their respective control.

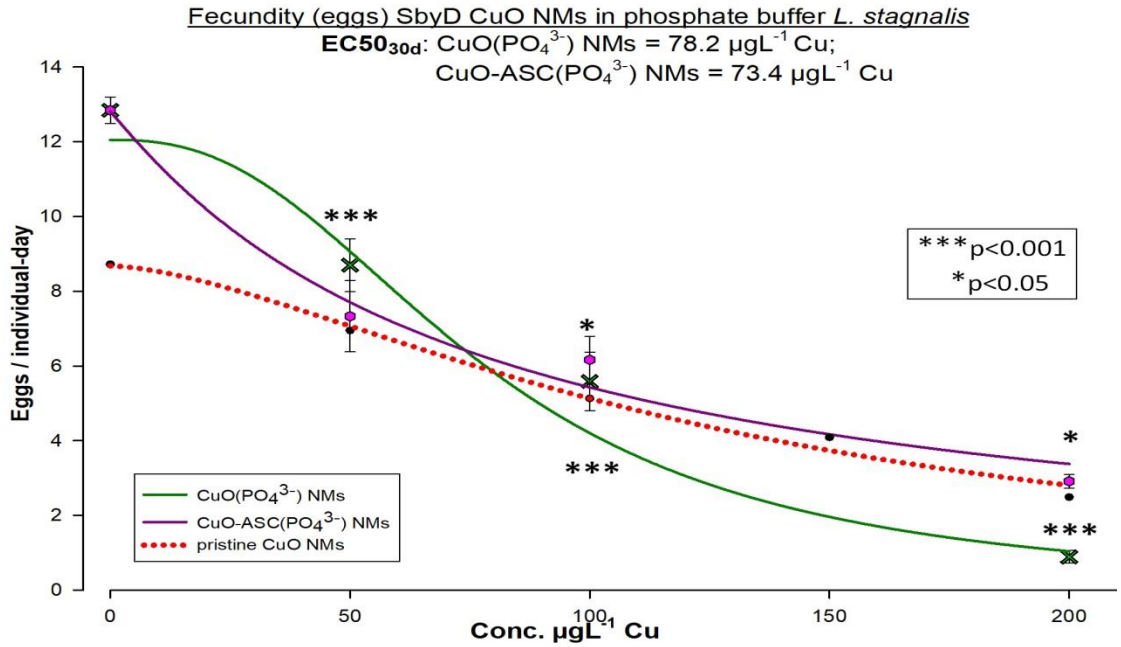

Figure 3-11 Cumulative number of eggs produced per individual-day over 30 d of exposure to $CuO(PO_4^{3-})$ NMs and $CuO\text{-}ASC(PO_4^{3-})$ NMs. For comparison, fitted model for snails exposed to up to 200 μgL^{-1} Cu of pristine CuO NMs is plotted, represented by the red dotted line (n = 4; error bars are SEM). Asterisks indicate significant differences, $p < 0.05$, compared with their respective control.

Surprisingly exposure to $CuO\text{-}PVP(PO_4^{3-})$ NMs elicited a greater toxicity than$CuSO_4$ and among all the CuO NMs tested as well. Since after 18 days of exposure to the highest concentration tested (200 μgL^{-1} Cu), mortality was caused to the 50% population, reproduction data were analysed only up to 15 days of exposure (Fig. 3-12 and 3-13). $EC50_{15d}$ calculated were 92.64 (± 8.49 SE) (Fig. 3-12) and 85.01 (± 11.49 SE) μgL^{-1} Cu (Fig. 3-13) for the cumulative number of clutches and eggs respectively.

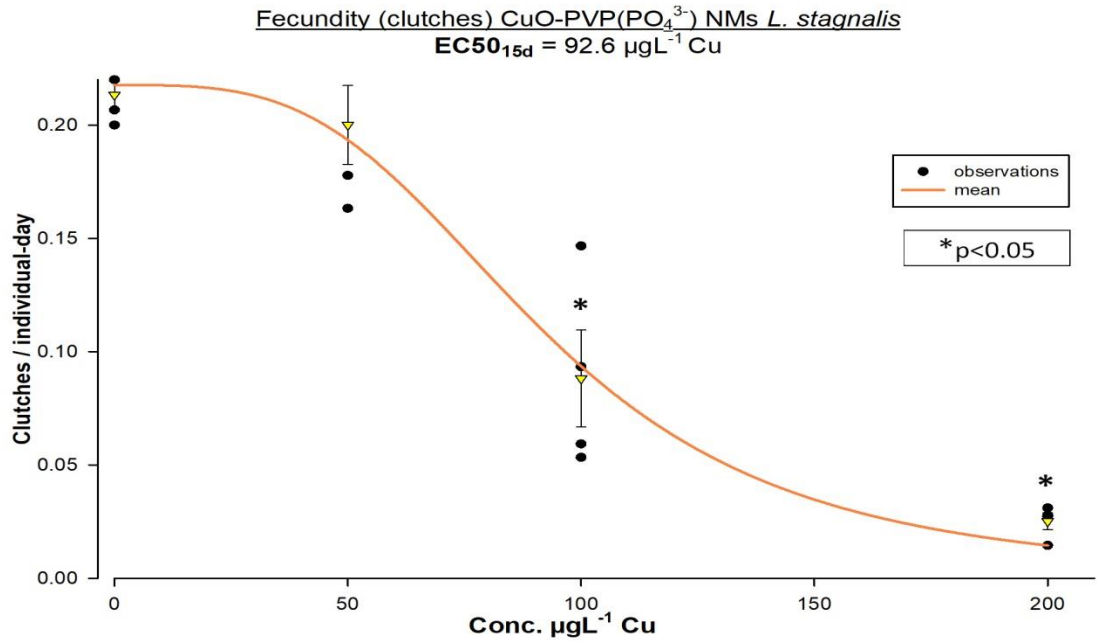

Figure 3-12 Cumulative number of clutches produced per individual snail per day over 15 d of exposure to CuO-PVP(PO_4^{3-}) NMs (n = 4; error bars are SEM). Solid lines stand for the fitted 4PL model; asterisks indicate significant differences, $p < 0.05$, compared with their respective control.

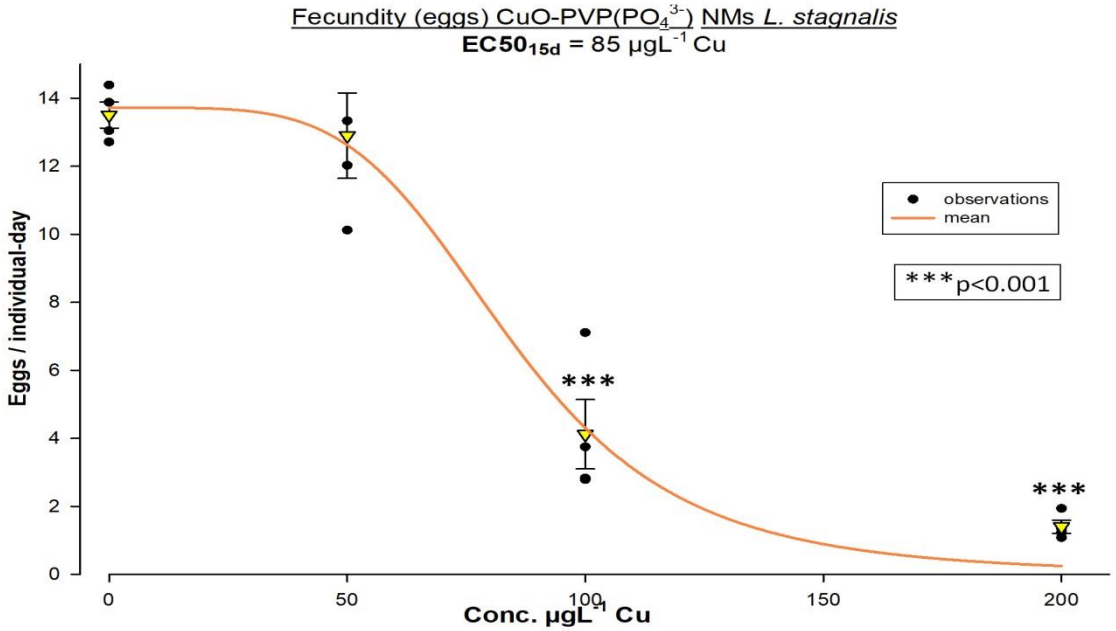

Figure 3-13 Cumulative number of eggs produced per individual snail per day over 15 d of exposure to CuO-PVP(PO_4^{3-}) NMs (n = 4; error bars are SEM). Solid lines stand for the fitted 4PL model; asterisks indicate a significant difference, $p < 0.05$, compared with their respective control.

3.3.2.3 Growth and food intake effects of aqueous Cu and CuO NMs on young adults

In agreement with the lethal results obtained, exposure to $CuSO_4$ resulted in a higher toxicity to young adult of *L. stagnalis* compared with pristine CuO NMs, expressed in

changing in weight over 30 days (Fig. 3-14 and 3-15) and feeding rate at 30 days (Fig. 3-16 and 3-17).

Snails exposed to $CuSO_4$ showed a marked reduction in weight in concentrations higher than 60 μgL^{-1} Cu (Fig. 3-14). Indeed, when checking the effect of concentration after 30 days of exposure, at the two highest concentrations tested (60 and 80 μgL^{-1} Cu) there was a significant decrease ($p < 0.05$) in weight compared with the control. At 30 days, a EC50 value of 70.35 (± 2.89) μgL^{-1} Cu was estimated. Multiple comparison between time points, however, indicated that there was no significant difference in the snail's growth across time points, yielding an F ratio of $F_{(4, 16)} = 0.06$, $p = 0.99$) (Fig. 3-14), due to the very high inter-snail variability.

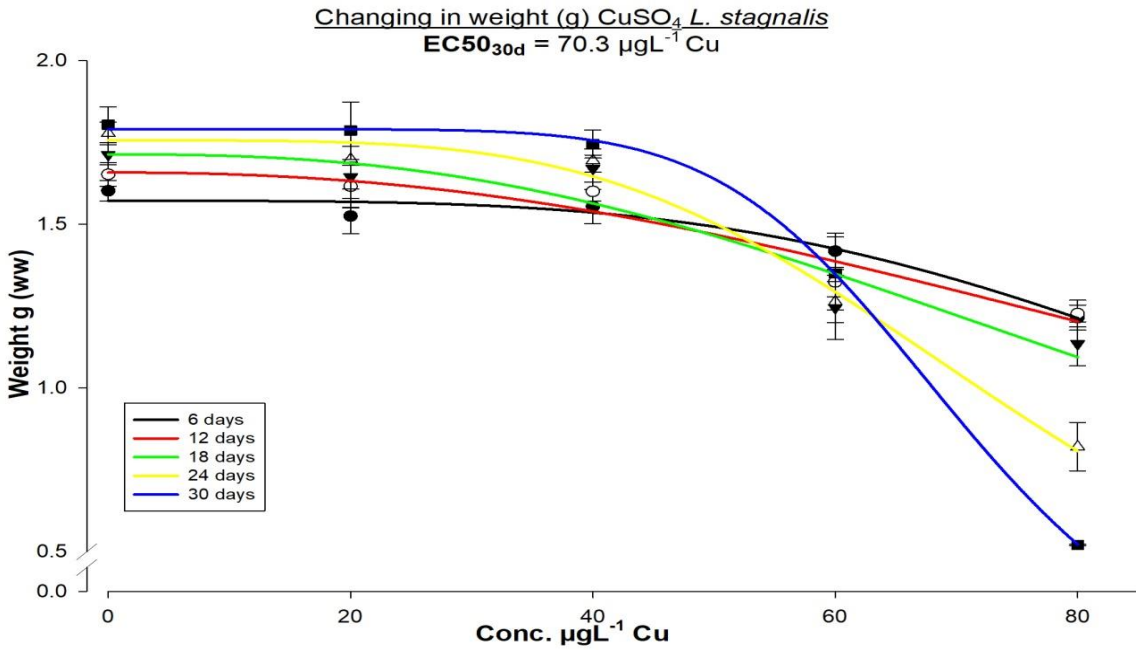

Figure 3-14 Changes in weight, expressed in grams as wet weight, of young adults of *L. stagnalis* (≈ 22 ± 2 mm) exposed to increasing concentrations of Cu as $CuSO_4$ at 20 ºC over 30 days. Solid lines stand for the fitted 4PL model (n = 4, error bars are SEM).

Snails exposed to concentration from 0 to 400 μgL^{-1} Cu as pristine CuO NMs exhibited a decrease in wet weight with concentrations and time (Fig. 3-15).

Fig. 3-15 shows that at concentrations of pristine CuO NMs higher than 200 μgL^{-1} Cu the weight wet of the snails decreased with long exposure time, while unexposed snails increased their weight considerably during the 30 days of the experiment. At 30 days of exposure only 20% of the population was affected, thus the EC20 calculated was 58.586 (±293.02 SE) μgL^{-1} Cu. A one-way RM ANOVA was conducted to compare the effect of time on weight of the exposed snails to pristine CuO NMs. There was no significant difference in weight with the increasing of time of exposure ($F_{(6, 24)} = 1.17$, $p = 0.35$).

However, a significant decrease in weight was revealed at 30 days between all the treatments and the control ($F_{(6, 21)}$ = 20.15, p < 0.001) (Fig. 3-15).

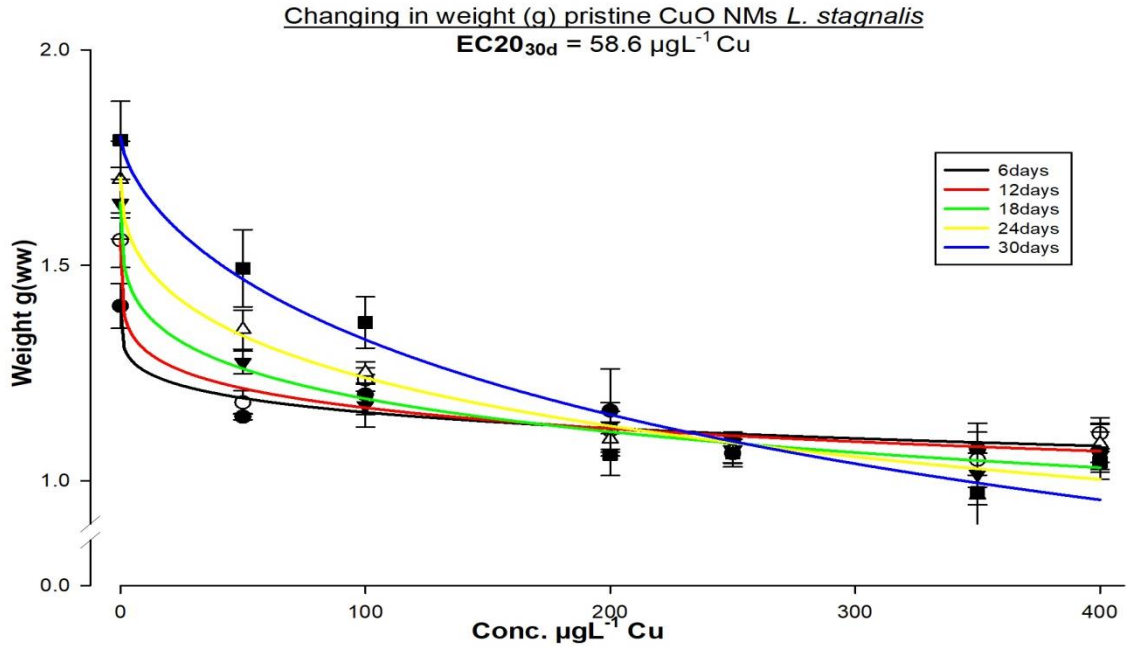

Figure 3-15 Changes in weight, expressed in grams as wet weight, of young adults of *L. stagnalis* (≈ 22 ± 2 mm) exposed to increasing concentrations of Cu as $CuSO_4$ at 20 ºC over 30 days. Solid lines stand for the fitted 4PL model (n = 4, error bars are SEM).

Findings gathered from the previous endpoint are in accordance with the response of feeding rate at 30 days due to exposure to respectively $CuSO_4$ and pristine CuO NMs.

Feeding rate for snails exposed to $CuSO_4$ was analysed only for snails exposed to up to 60 µgL^{-1} Cu (Fig. 3-16) as an insufficient amount of snails was present at the highest concentration tested (80 µgL^{-1} Cu) to perform the feeding rate test, due to the high mortality observed (Fig. 3-3). Results indicated that snails were affected at concentrations higher than 40 µgL^{-1} Cu with an EC50 value estimated as 44.78 (± 6.73 SE) µgL^{-1} Cu. A one-way ANOVA between different concentrations was conducted to compare the effect of concentrations of $CuSO_4$ on the feeding behaviour of the snails. There was a significant inhibition on the consumption of lettuce per individual per day for the four treatments ($F_{(3, 10)}$ = 23.1, p < 0.001). Post hoc comparisons using the Dunnett's method indicated that the mean score for the control was significantly different only from the highest concentration tested of 60 µgL^{-1} Cu (p < 0.001). No significant differences were found between the control and the other two treatments of 20 µgL^{-1} Cu (p = 0.15) and 40 µgL^{-1} Cu (p = 0.11) (Fig. 3-16).

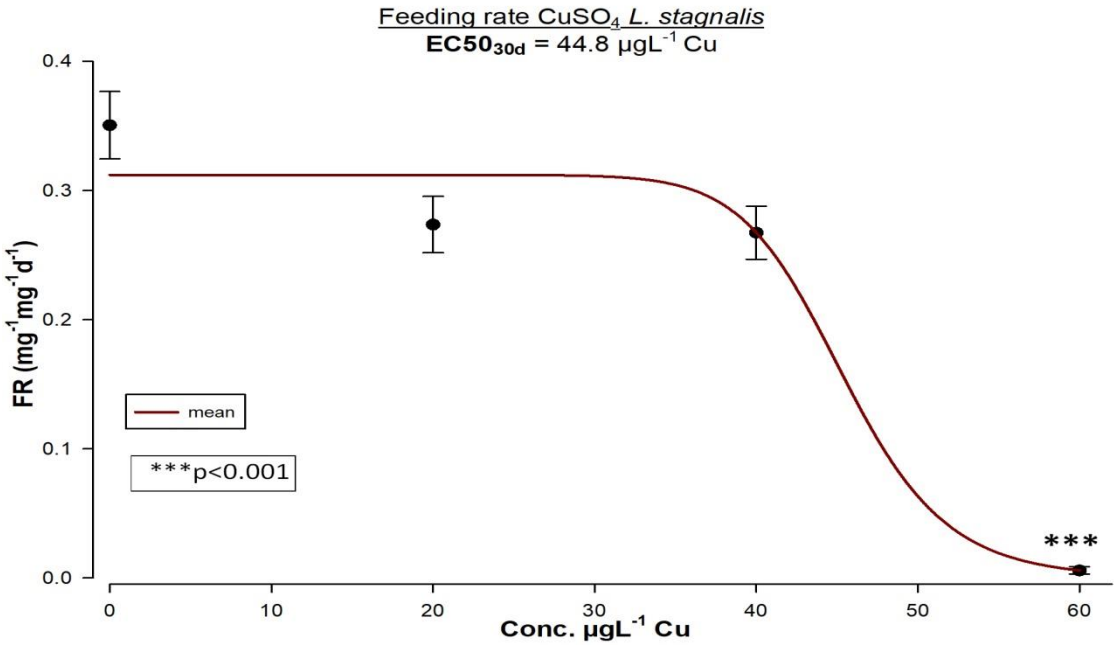

Figure 3-16 Feeding rate expressed in mg of lettuce consumed per day normalized per mg of wet weight of young adults of *L. stagnalis* (≈ 22 ± 2 mm) exposed to increasing concentrations of Cu as $CuOSO_4$ at 20 ºC for 24 hrs after 30 days of exposure (n = 3, error bars are SEM). Solid lines stand for the fitted 4PL model; asterisks indicate significant differences, $p < 0.05$, compared with the control.

During the 30 days of exposure to pristine CuO NMs, a decrease on the feeding rate and excretion was observed in concentration-response manner. After one week, snails exposed to concentration of pristine CuO NMs higher than 150 μgL^{-1} Cu stopped eating and excreting. Indeed, results from the feeding rate calculated after 30 days of exposure revealed an EC50 of 157 (± 59.03 SE) μgL^{-1} Cu (Fig. 3-17). Analysis of variance (ANOVA) indicated that food consumption was significantly ($p < 0.05$) reduced with the increasing of exposure concentration ($F_{(3,\ 8)} = 28.59$, $p < 0.001$). Post hoc comparisons using the Dunnett's Method indicated that the mean score for control was significantly different from the treatments at concentrations of 250 μgL^{-1} Cu and 350 μgL^{-1} Cu ($p < 0.001$). However, no significant difference was found in food consumption between the lowest concentration tested, 100 μgL^{-1} Cu, and the unexposed snails ($p = 0.33$) (Fig. 3-17).

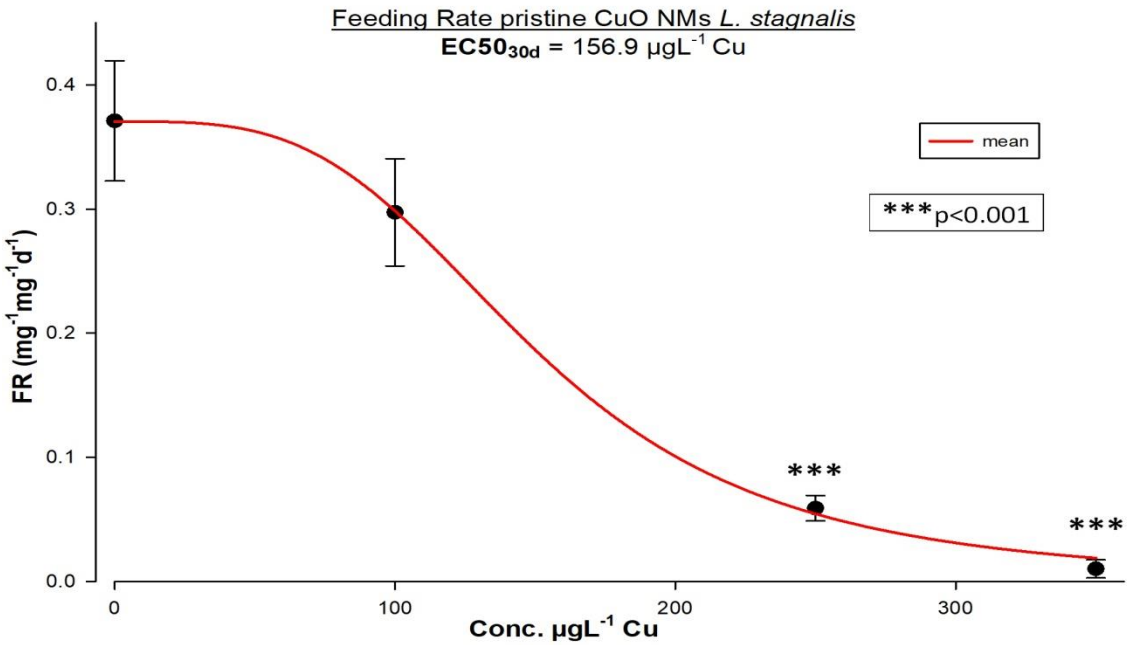

Figure 3-17 Feeding rate expressed in mg of lettuce consumed per day normalized per mg of wet weight of young adults of *L. stagnalis* (≈ 22 ± 2 mm) exposed to increasing concentrations of Cu as pristine CuO NMs at 20 ºC for 24 hrs after 30 days of exposure (n = 3, error bars are SEM). Solid lines stand for the fitted 4PL model; asterisks indicate significant differences, $p < 0.05$, compared with their respective control.

Findings from experiments with SbyD CuO NMs revealed a more moderate effect on young adult's snail growth, except for those exposed to CuO-PVP(PO_4^{3-}) NMs (Fig. 3-18 and 3-19 and Fig. B-1, B-2. B-3, B-4 and B-5 in Appendix B).

Consistent with the fecundity endpoints, results from exposure to SbyD CuO NMs functionalised in Milli-Q water elicited one of the lowest ECx among all the CuO NMs tested. Indeed, at 30 days it was possible to estimate only an EC20 of 132.34 (± 57.689 SE) µgL^{-1} Cu for snails exposed to CuO-ASC(H_2O) NMs, which it is about 2 times lower than the EC20 calculated for exposure to pristine CuO NMs (EC20 = 72.42 (± 315 SE) µgL^{-1} Cu) at the same concentration range, 0-200 µgL^{-1} Cu (plotted in Fig. 3-18 as a red dotted line). Indeed, snails exposed to CuO-PVP(H_2O) NMs seemed unaffected by the NMs. Indeed, although a slight decrease in weight at 25 µgL^{-1} Cu was observed, there were no differences between the other higher concentrations and the control (Fig. 3-18 and Fig. B-2 in Appendix B for further details). Thus, it was not possible to estimate an ECx after 30 days of exposure.

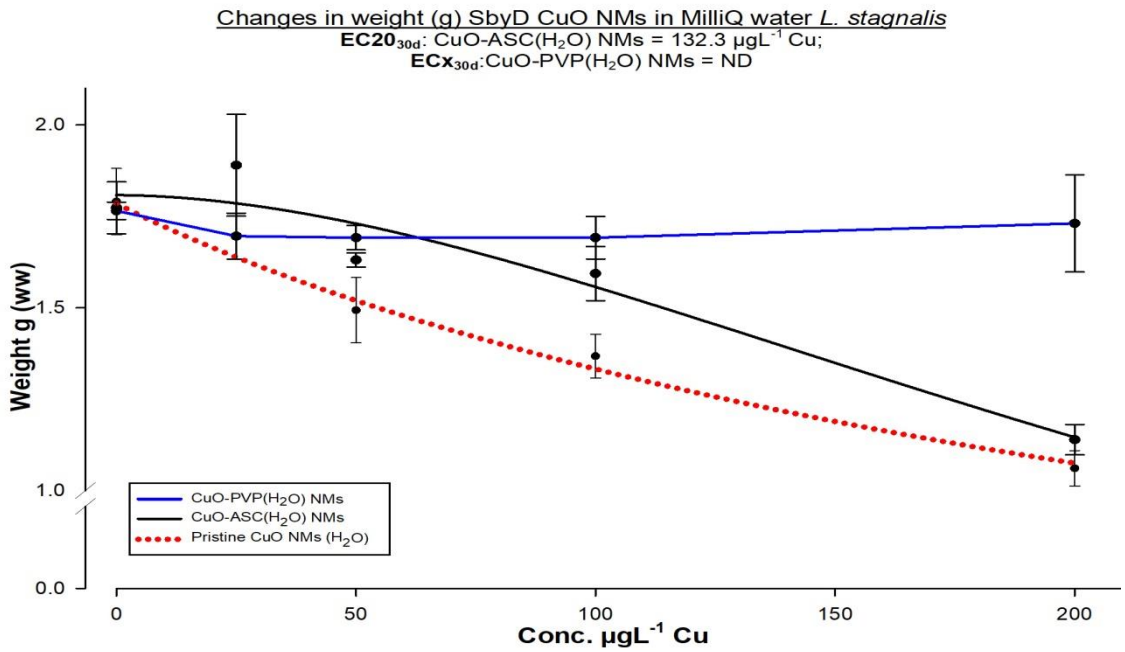

Figure 3-18 Changes in weight, expressed in grams as wet weight, of young adults of *L. stagnalis* (≈ 22 ± 2 mm) exposed at 20 °C for 30 days to increasing concentrations of SbyD CuO NMs functionalised in Milli-Q water. Black line represents the fitted model for snails exposed to CuO-ASC(H_2O) NMs; blue line represents a line per point for snails exposed to CuO-PVP(H_2O) NMs. Furthermore, for comparison, the fitted model for snails exposed to up to 200 µgL^{-1} Cu of pristine CuO NMs is plotted, represented by the red dotted line (n = 4, error bars are SEM.)

In contrast, exposure to CuO-PVP(PO_4^{3-}) NMs resulted in the highest inhibition of the growth of exposed snails among all the CuO NMs investigated. Indeed, snails grew significantly less than the control at the two highest concentration tested of 100 µgL^{-1} Cu and 200 µgL^{-1} Cu ($p < 0.05$), with an EC50 calculated at 30 days of 166.292 (± 26.482 SE) µgL^{-1} Cu (Fig. 3-19). A less severe effect on growth compared with the pristine CuO NMs was recorded for snails exposed to CuO-ASC(PO_4^{3-}) and CuO(PO_4^{3-}), with at 30 days the EC20 estimated as 168.51 (±59.36 SE) µgL^{-1} Cu and 70.35 (± 67.84 SE) µgL^{-1} Cu respectively (Fig. 3-19).

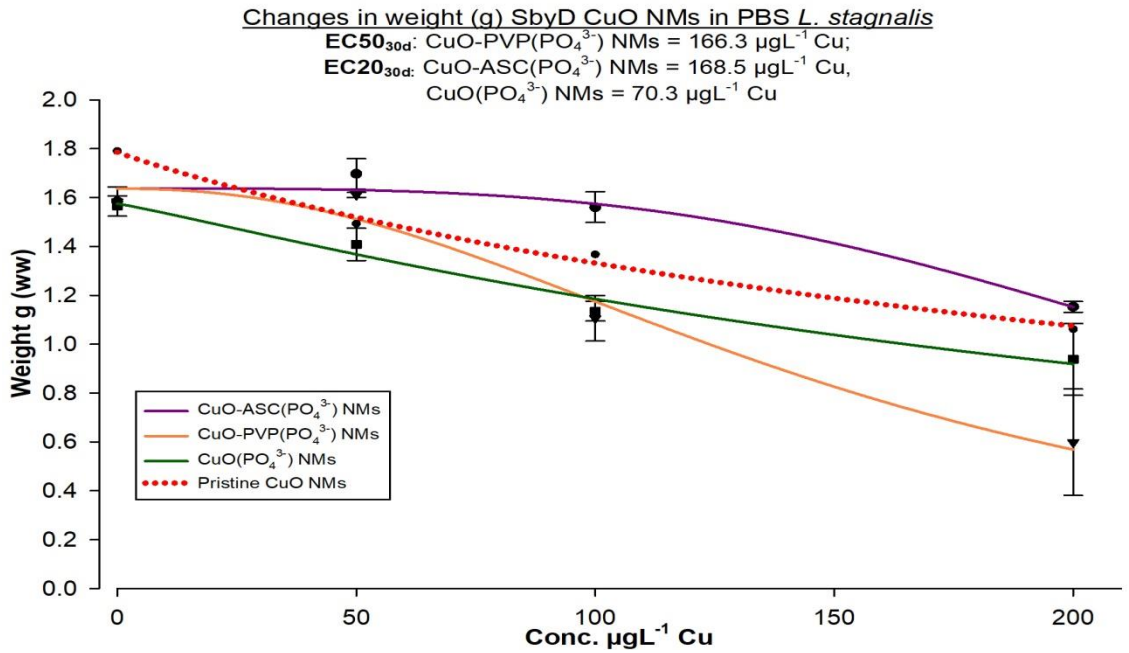

Figure 3-19 Changes in weight, expressed in grams as wet weight, of young adults of *L. stagnalis* (≈ 22 ± 2 mm) exposed at 20 °C for 30 days to increasing concentrations of SbyD CuO NMs functionalised in PBS. Solid lines stand for the fitted 4PL model. For comparison, the fitted model for snails exposed to up to 200 µgL^{-1} Cu of pristine CuO NMs is plotted, represented by the red dotted line (n = 4, error bars are SEM).

In disagreement with the growth endpoint, exposure to SbyD CuO NMs induced a different toxicity ranking on the feeding behaviour of young adults of *L. stagnalis*. Snails exposed to CuO-ASC(H_2O) NMs exhibited the highest feeding rate inhibition among all the CuO NMs investigated (Fig. 3-20, 3-21 and 3-22). The EC50 value estimated was 58.89 (± 9.76 SE) µgL^{-1} Cu (Fig. 3-21). In contrast, but in agreement with the growth and fecundity endpoints, exposure to CuO-PVP(H_2O) NMs did not induced any significant changes in the feeding behaviour of the snails, thus no ECx could be calculated (Fig. 3-20).

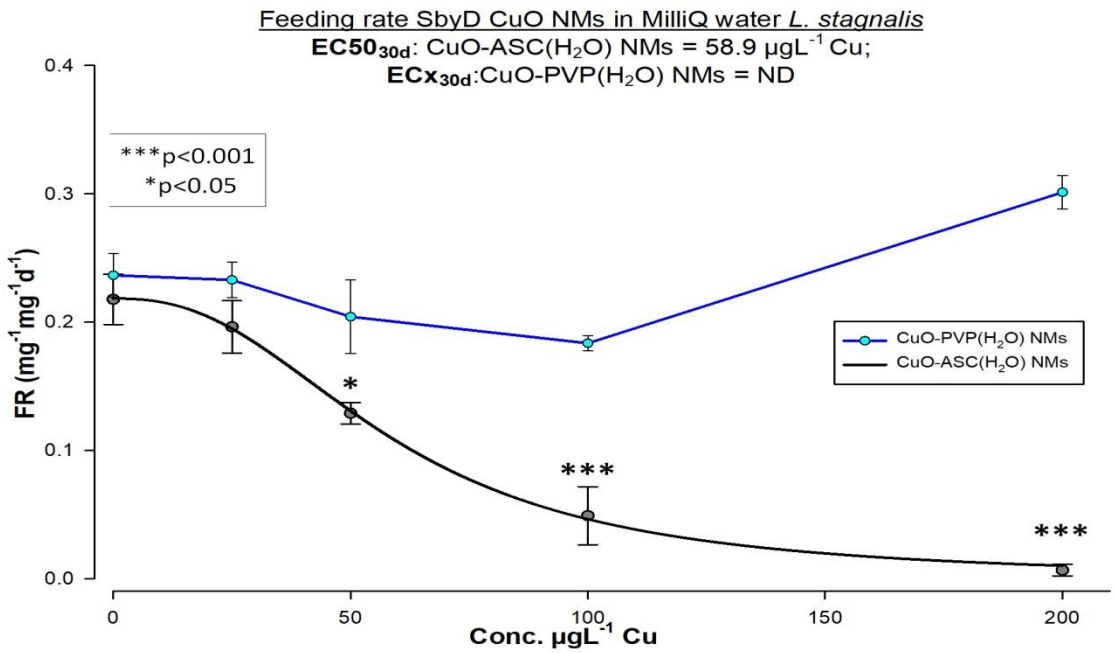

Figure 3-20 Feeding rates expressed as grams of lettuce consumed per day normalised per mg of wet weight of young adults of *L. stagnalis* (≈ 22 ± 2 mm) exposed to increasing concentrations of SbyD CuO NMs functionalised in Milli-Q water, at 20 °C, for 24 hrs after 30 days experiment. The black line represents the fitted model for snails exposed to CuO-ASC(H_2O) NMs; blue line represents a line per point for snails exposed to CuO-PVP(H_2O) NMs (n = 3, error bars are SEM). Asterisks indicate significant differences compared with their respective controls ($p < 0.05$).

Results gathered from exposure to SbyD CuO NMs functionalised in phosphate buffer revealed a similar toxicity ranking to the fecundity endpoints, where CuO-PVP(PO_4^{3-}) NMs were found to be the most toxic among the 3 different NMs. A moderate reduction in feeding was recorded at concentrations above 70 μgL^{-1} Cu (Fig. 3-21) for CuO-ASC(PO_4^{3-}) NMs and CuO$(PO_4)^{3-}$ NMs, with EC50s calculated respectively of 74.03 (± 16.57 SE) μgL^{-1} Cu and 82.91 (± 15.37 SE) μgL^{-1} Cu (Fig. 3-21). In contrast, due to high mortality, feeding rate was not calculated for snails exposed to 200 μgL^{-1} Cu of CuO-PVP(PO_4^{3-}) NMs. However, a significant decrease in food consumption was recorded already at the concentration of 100 μgL^{-1} Cu, resulting in an EC50 of 61.14 (± 18.99 SE) μgL^{-1} Cu (Fig. 3-22).

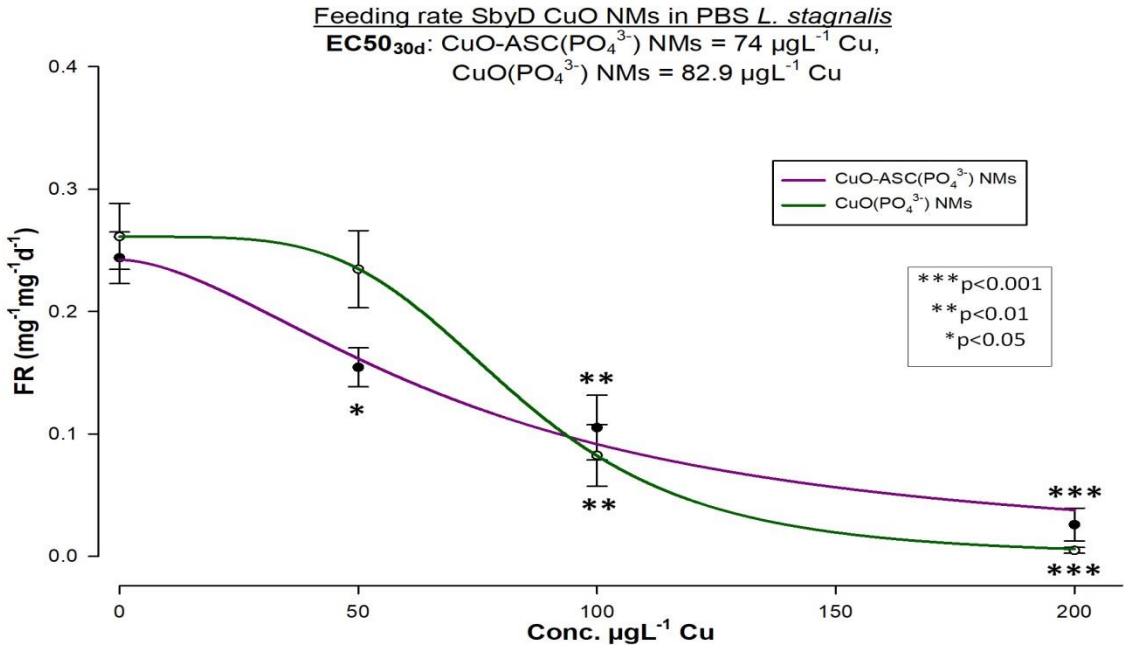

Figure 3-21 Feeding rate expressed as mg of lettuce consumed per day normalised per mg of wet weight of young adults of *L. stagnalis* (≈ 22 ± 2 mm) exposed to increasing concentrations of CuO-ASC(PO_4^{3-}) NMs and CuO(PO_4^{3-}) NMs for 24 hrs, at 20 ºC, after 30 days experiment(n = 3, error bars are SEM). Solid lines stand for the fitted 4PL model; asterisks indicate significant differences, $p < 0.05$, compared with their respective controls.

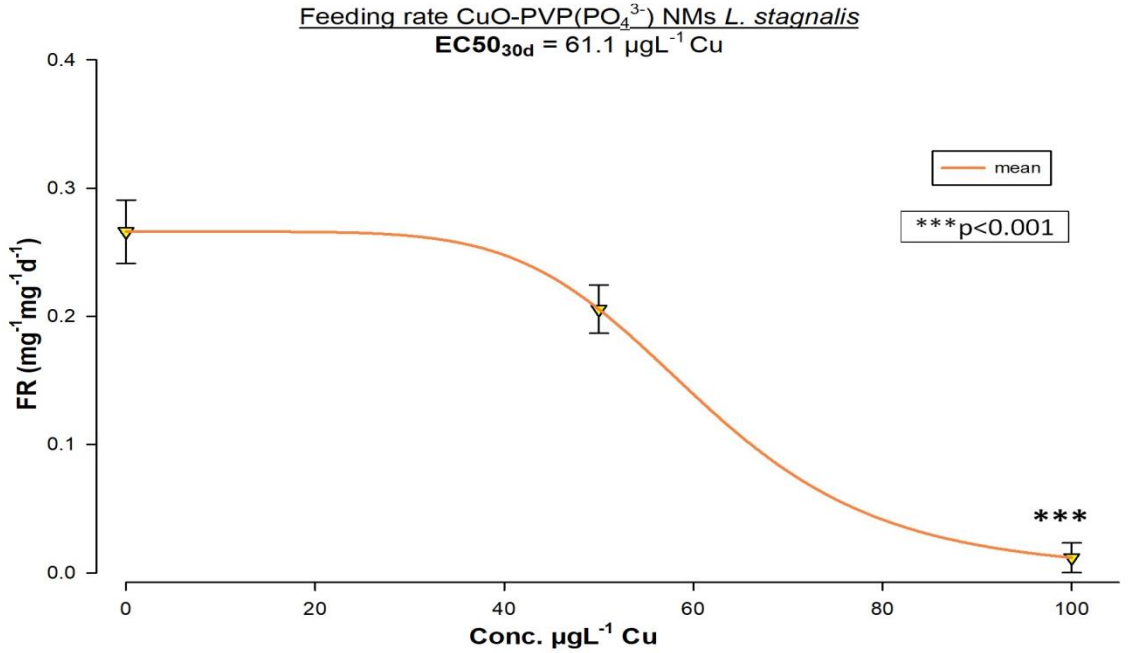

Figure 3-22 Feeding rate expressed as mg of lettuce consumed per day normalised per mg of wet weight of young adults of *L. stagnalis* (≈ 22 ± 2 mm) exposed to increasing concentrations of CuO-PVP(PO_4^{3-}) NMs, at 20 ºC, for 24 hrs after30 days experiment(n = 3, error bars are SEM). Asterisks indicate significant differences, $p < 0.05$, compared with the control.

Table 3-3 summarises the results gathered from all the endpoints evaluated during the chronic exposure to Cu as ionic Cu and CuO NMs.

Table 3-3 Summary of the results, L/ECx values of mortality, fecundity, growth and feeding rate endpoints after chronic exposure to ionic Cu and CuO NMs tested.

	L/ECx (30 days)				
	Mortality %	**Fecundity**		**Weight g (ww)**	**Feeding rate (mg g^{-1} d^{-1})**
		Clutches/day	**eggs/day**		
$CuSO_4$	LC50: 63.5 μgL^{-1} Cu	EC50: 63 μgL^{-1} Cu	EC50: 49 μgL^{-1} Cu	EC50: 70 μgL^{-1} Cu	EC50: 45 μgL^{-1} Cu
Pristine CuO NMs (H_2O)	LC50: 492 μgL^{-1} Cu	EC50: 157 μgL^{-1} Cu	EC50: 132 μgL^{-1} Cu	EC20: 59 μgL^{-1} Cu	EC50: 157 μgL^{-1} Cu
CuO-PVP(H_2O) NMs	n.d.	n.d.	n.d.	n.d.	n.d.
CuO-ASC(H_2O) NMs	n.d.	EC50:133 μgL^{-1} Cu	EC50: 77 μgL^{-1} Cu	EC20: 132 μgL^{-1} Cu	EC50: 59 μgL^{-1} Cu
CuO(PO_4^{3-}) NMs	LC30: 186 μgL^{-1} Cu	EC50: 116 μgL^{-1} Cu	EC50: 78 μgL^{-1} Cu	EC20: 70 μgL^{-1} Cu	EC50: 83 μgL^{-1} Cu
CuO-PVP(PO_4^{3-}) NMs	LC50: 160 μgL^{-1} Cu	EC50 (15days): 93 μgL^{-1} Cu	EC50 (15days): 85 μgL^{-1} Cu	EC50: 166 μgL^{-1} Cu	EC50: 61 μgL^{-1} Cu
CuO-ASC(PO_4^{3-}) NMs	n.d.	EC50: 114 μgL^{-1} Cu	EC50:73 μgL^{-1} Cu	EC20: 168 μgL^{-1} Cu	EC50: 74 μgL^{-1} Cu

3.4 Discussion

Different studies have demonstrate the high sensitivity of *L. stagnalis* to heavy metals chronic exposure (Grosell et al. 2006, Grosell and Brix 2009, Brix et al. 2011, Brix et al. 2012, Munley et al. 2013, Niyogi et al. 2014), indicating mortality or growth as the most sensitive endpoints to be affected (Niyogi et al. 2014). However, no study has investigated the chronic ecotoxicity of CuO NMs.

It is within this knowledge gap that this research study is placed, aiming to investigate the chronic lethal and sublethal toxicity of different CuO NMs (pristine and SbyD CuO NMs) on young adults of *L. stagnalis* used as model test species of the freshwater benthic ecosystem. In particular, in light of the recent approval by the OECD of this species as a test model for the mollusc reproduction test (OECD 2016), reprotoxicity endpoints and effect on growth and feeding consumption were evaluated.

Initial chronic studies were conducted to investigate, for the first time, the comparative lethal and sublethal toxicity of aqueous Cu as $CuSO_4$ and pristine CuO NMs, as baseline

for the experiments using the SbyD CuO NMs. Overall, findings suggested that as Cu concentrations and exposure time increases; the fecundity, growth and feeding intake decreases, thus indicating concentration-dependent responses (Tab. 3-3).

The chronic lethality results, in agreement with the acute studies, indicated a greater toxicity of the ionic Cu compared with the pristine CuO NMs. LC50 values estimated, at 30 days, were 63 (Fig. 3-1) and 492 μgL^{-1} Cu (Fig. 3-2) for respectively $CuSO_4$ and pristine CuO NMs. Results from $CuSO_4$ exposures are in line with Ng et al. (2011), where no mortality was observed in chronic exposures of younger *L. stagnalis* (size range 12–15 mm) to concentration up to 35 μgL^{-1} Cu as $CuSO_4$. In this study, snails' mortality increased with concentration from 40 μgL^{-1} Cu, reaching 80% mortality at the highest concentration tested of 80 μgL^{-1} Cu.

Results from the characterisation studies revealed that, simulating the experimental conditions (3 days exposure at 20° C and 16/8h light cycle), NMs were stabilized in comparison to samples analysed in standard conditions at 24h. Indeed, a considerable decrease (up to 6-fold, depending on the NMs) of the HDD was reported across all the CuO NMs, regard of the coating or dispersant used (Tab.3-1 and 3-2), while particle dissolution was enhanced in this diluted condition for all NMs. No significant changes in the ζ-potential values, which ranged between -6 and -13 mV, were reported. This remarkable increase in dissolution with exposure time has been reported also by Shi et al. (2011), where CuO NMs dissolution increased with time over a two-day period reaching an 80% within 48 h.

The higher dissolution would suggest that, as other authors proposed (Pradhan et al. 2012), the chronic toxicity of the pristine CuO NMs was likely driven by the high quantity of ions in solution present after 3 days of exposure. However, if this was true we should have seen proportional direct toxicity with increased dissolution of the different CuO NMs, which was recorded in this research study (Tab. 3-3). Indeed, SbyD CuO NMs functionalised in phosphate buffer presented a lower dissolution of about 30 % compared with those in Milli-Q water which was about 70% (Tab. 3-1). Nevertheless, when comparing the chronic lethal toxicity of all the SbyD CuO NMs tested at sublethal concentrations (0-200 μgL^{-1} Cu, based to the LC50 of pristine CuO NMs), CuO-PVP(PO_4^{3-}) NMs and CuO(PO_4^{3-}) NMs exposures exhibited the highest mortality after 30 days among all the CuO NMs investigated, with an LC30 estimated as 186 μgL^{-1} Cu for exposure to CuO(PO_4^{3-}) NMs and LC50 of 160 μgL^{-1} Cu for experiments with CuO-PVP(PO_4^{3-}) (Fig. 3-3).

These data are in agreement with Líbalová et al. (2018) cytotoxicity ranking of the same SbyD CuO NMs, functionalised in phosphate buffer, to a mouse macrophage cell line. Cell exposed to CuO-PVP(PO_4^{3-}) produced the highest level of ROS, while those exposed to CuO-ASC(PO_4^{3-}) produced the opposite result. Authors consequently were not able to directly link the toxicity of the SbyD CuO NMs only to NMs dissolution and subsequent Cu burden in cells or to ROS production. Similarly, in this research study, exposure to SbyD CuO NMs modified with ASC (either dispersant agent) resulted in the lowest lethal chronic mortality at 30 days.

It is important also to note that dissolution analysis, although simulating experimental condition, were performed in abiotic condition in the absence of organisms and food. The exposure environment can be critical in defining their toxicity. For example, numerous authors have reported the mitigating toxicity effect of NOM to Cu exposure complexing copper and reducing the bioavailable copper ion concentrations (Santore et al. 2001, Blinova et al. 2010, Jiang et al. 2017, Peng et al. 2017). This reduction in toxicity in the presence of NOM was demonstrated by Blinova et al. (2010) evaluating their influence in the toxicity of CuO NMs and $CuSO_4$ to the crustacean *D. magna*. Interestingly, results showed that the mitigation effect was more remarkable in experiments with CuO NMs, where a significant difference in mortality was revealed between water with high (lower mortality) and low NOM concentration (higher mortality), due to lower bioavailability of the dissolved ions to the crustacean.

This mechanism could have played a significant role in the results gathered by this research study. Experiments were conducted in the presence of food (lettuce *at libitum*) which is likely to have increased the presence of dissolved organic matter in the exposure vessel produced by the deterioration of the uneaten lettuce leaves and the excretion of the snails for 3 days (at which time a 100% renewal of the medium was performed). The extracellular organic matter could have coated the CuO NMs, inhibiting ion release by preventing interaction of the NMs surface with the exposure medium and reducing the availability of H^+ (Wu et al. 2017). Furthermore, the speciation forms of soluble Cu ions (Cu, Cu(II) Cu(I) $CuCl^+$, etc.) may considerably change within the range of physiological conditions used in this study depending not only on small variations of pH but also on anions as well as redox potential (Lowry et al. 2012). Finally, as previously demonstrated (Ng et al. 2011, Pradhan et al. 2012), Cu ions may also bind to the food left in the exposure vessel and be taken up by snails from the diet.

Thus, it is clear that the mechanisms of toxicity involving all the CuO NMs tested are more complex and cannot be solely explained by dissolution data. The hydrodynamic size and surface charge of NMs dispersions might have also affected the way in which the organisms responded upon exposure (Jiang et al. 2009).

Results from the sublethal endpoints confirm the hypothesis that the high dissolution of the CuO NMs was not the determining (or sole) factor of toxicity (Tab. 3-2 and 3-3).

Indeed, over 30 days of exposure, among all the CuO NMs tested and $CuSO_4$, CuO-PVP(PO_4^{3-}) NMs, which reported the lowest dissolution rate (29%), caused the highest fecundity inhibition. Given the significant mortality at the highest concentration tested (200 μgL^{-1} Cu), fecundity endpoints were calculated only after 15 days of exposure with $EC50_{15d}$ values estimated as 97 (Fig. 3-12) and 83 μgL^{-1} Cu (Fig. 3-13) for the cumulative number of clutches and eggs respectively. In contrast, snails exposed to CuO-PVP(H_2O) NMs showed the lowest toxicity among all the sublethal endpoints evaluated (fecundity, growth and feeding rate) (Fig. 3-8, 3-9, 3-18, 3-20 and B-2). Fecundity data showed that snails were only initially slightly affected by the exposure to CuO-PVP(H_2O) NMs, showing a small decrease in cumulative numbers of clutches and eggs already at 25 μgL^{-1} Cu, but with no further reduction with the increasing of exposure concentrations and time (Fig. 3-8 and 3-9). Other studies have demonstrated the reduction in toxicity of NMs coated with PVP due to the stabilizing effect of the latter (Baumann et al. 2014, Hou et al. 2017b). Thus, the opposite toxicity behaviour, observed in this study, between the two SbyD CuO NMs coated with PVP, suggests that the presence of the phosphate buffer might have played a significant role in the toxicity of the CuO-PVP(PO_4^{3-}) NMs.

Li et al. (2011) evaluating the influence of medium components in the toxicity of ZnO NMs to *Escherichia coli* found that the presence of PBS in the exposure medium contributed on reducing strongly the toxicity of the ZnO NMs, nevertheless the high NMs dissolution. These decrease in toxicity was attributed to the generation of precipitates $Zn_3(PO_4)^2$ and zinc complexes, which significantly decreased the concentration of Zn^{2+} ions in solution.

It is possible that in this research study, Cu-phosphate precipitates were also formed in the exposures of SbyD CuO NMs functionalised in PBS, reducing the concentration of Cu ions in solution. However, contrary to Li et al. (2011), snails being benthic feeder grazed on the precipitates increasing the bioavailability of the NMs, hence the higher toxicity.

A similar inhibition in the number of cumulative clutches over 30 days was induced by the other CuO NMs, with EC50 values estimated to be 157, 117, 114 and 138 μgL^{-1} Cu for respectively pristine CuO NMs (Fig. 3-6), CuO(PO_4^{3-}) NMs (Fig. 3-10), CuO-ASC(PO_4^{3-}) NMs (Fig. 3-10) and CuO-ASC(H_2O) NMs (Fig. 3-8). The same trend was observed in the eggs endpoint, with exception of the pristine CuO NMs, where a weaker inhibition was shown in the cumulative numbers (EC50: 132 μgL^{-1} Cu) (Fig. 3-7) compared with the other aforementioned NMs (EC50: CuO(PO_4^{3-}) NMs 79 μgL^{-1} Cu (Fig. 3-11), CuO-ASC(PO_4^{3-}) NMs 73 μgL^{-1} Cu (Fig. 3-11); CuO-ASC(H_2O) NMs 75 μgL^{-1} Cu (Fig. 3-9)).

Fecundity findings of snails exposed to $CuSO_4$ showed a similar toxicity behaviour observed in Das and Khangarot (2011). These authors exposing adults of *Lymnaea luteola* to increasing concentrations of $CuSO_4$ noted, like in this study, at low exposure concentrations of 10 μgL^{-1} Cu, a decrease in clutches' size and in numbers of eggs. With the increase in exposure concentration, empty egg masses were produced more frequently (Das and Khangarot 2011). Results from this research study of snails exposed to $CuSO_4$ revealed a significant reduction in the production of eggs at the lowest concentration tested (20 μgL^{-1} Cu), but a significant effect in the clutches production only at the two highest concentrations of 60 and 80 μgL^{-1} Cu (Fig. 3-4 and 3-5). EC50 values estimated were 63 and 49 μgL^{-1} Cu for, respectively, clutches and eggs endpoints.

In order to understand if there was a relationship between the fecundity inhibition and the observed decrease in growth and feeding rate observed in snails exposed to $CuSO_4$ at 30 days, a multiple Pearson's correlations were computed. Analyses revealed a stronger, positive significant correlation between the food consumed and the decrease in number of eggs produced ($r = 0.989$, $n = 4$, $p = 0.011$) compared with clutches ($r = 0.970$, $n = 4$, $p = 0.0302$). Furthermore, a strong positive correlation, which was also significant, was shown also between the changing in weight and the feeding rate ($r = 0.984$, $n = 4$, $p = 0.016$). Indeed, EC50 estimates for feeding rate and changing in weight at 30 days was respectively 45 and 70 μgL^{-1} Cu. Thus, it appeared that snails were able to detect Cu in the test medium, at concentrations over 40 μgL^{-1} Cu, and therefore reduced food consumption which directly affected the fecundity of the snails. Snails were still reproducing laying egg masses but with a considerable reduction in the number of eggs per clutch. In addition, a dropping in weight with the extending of the exposure time was registered at concentration over 60 μgL^{-1} Cu (Fig. 3-14), which coincide with the effect on 50 % of population in the number of the cumulative clutches

laid over 30 days (Fig. 3-4). This relationship was observed also by Das and Khangarot (2011) study, where *L. luteola* spent the first 2 weeks of the experiment, accumulating Cu from the test solution. As result, Cu reached high concentrations inside the snails, causing them to reduce or stop feeding before toxicity took place. This phenomenon might be attributed to a redirection of energy from growth to reproduction, as previous studies showed in the Florida apple snail, *Pomacea paludosa*, which exhibited a decreased in growth rates due to exposure to ionic Cu (Rogevich et al. 2009). In contrast, Ng et al. (2011) exposing juveniles of *L. stagnalis* at sublethal concentrations (up to 32 μgL^{-1} Cu) of $CuSO_4$, did not attribute the inhibition of the feeding rate to the decrease in weigh as after 4 weeks snails were not significantly affected in the food they consumed but they significantly decreased in weight. However, this contrasting conclusion can be explained by the smaller size of snails and lower concentration range used in Ng et al. (2011) compared with this research study.

Results from experiments with pristine CuO NMs revealed the same correlation between feeding consumption, changing in weight and fecundity. 50% of the snail population reduced significantly the food consumption (Fig. 3-17) and clutches laying (Fig. 3-6) at the same exposure concentration of 157 μgL^{-1} Cu, with and earlier effect in the eggs production (EC50 132 μgL^{-1} Cu) (Fig. 3-7). This directly reflected in the capability of the snails to gain weight over time, although less dramatically than exposure to $CuSO_4$. Indeed, at concentration exceeding 200 μgL^{-1} Cu snails appeared to lose weight, as they got older due to the apparent blockage of the digestive functions deducted by the lack of faeces in the exposure vessel. However, after 30 days of exposure only 20% of the population decreased significantly in weight, with an EC20 value estimated as 59 μgL^{-1} Cu (Fig. 3-15).

Croteau et al. (2014b) studied the bioaccumulation of CuO NMs after 5h of waterborne or feeding exposure to adults of *L. stagnalis*. Authors demonstrated that the uptake rate of snails exposed to waterborne ionic Cu from solution was two-fold greater than snails in the CuO NMs test. In contrast, when snails were exposed to spiked food (diatoms), CuO NMs uptake rate exceeded that of Cu-laden diatoms, suggesting that CuO NMs were less bioavailable to *L. stagnalis* than Cu added as $Cu(NO_3)_2$. These results corroborate the assertion that dissolution was not the main driver of the overall toxicity of the CuO NMs under these experimental conditions.

Blinova et al. (2010) observed and quantified the accumulation of CuO NMs in the gut of *D. magna*. After exposure to either sub-lethal or lethal concentrations of CuO NMs, the gut of daphnids was visible filled with CuO NMs, indicating that the toxicity

observed was due to the dissolved Cu ions from the NMs and not to the gut-accumulated NMs, since no correspondence in mortality was seen. However, in their study, organisms were still excreting during the whole experiment time, thus the same conclusion cannot be drawn here, where a good correlation was found between the feeding rate and decrease in weight. It is also possible that some of the suspended copper complexes formed with DOM were more bioavailable to the crustacean than to the snails in this research study, due the different feeding behaviour of the two organisms; since daphnids are filter feeders and snails are substrate grazers (Apte et al. 2006).

Overall findings from all the sublethal endpoints in the SbyD CuO NMs exposures confirmed the hypothesis of a direct link between the feeding rate inhibition and the decrease on the number of laid clutches and in a more significant manner, in egg production. In fact, when snails were exposed to CuO-PVP(H_2O) NMs they did not substantially alter their feeding rate in response to increasing concentrations of the NMs, with no concentration-response was recorder for the reproduction and growth endpoint. It is worth of notice, that for both fecundity endpoints, the cumulative numbers of clutches/eggs were however significant different from the control at the lowest concentration of 25 μgL^{-1} Cu reaching, however, a plateau with the increasing concentrations. This suggests that the presence of PVP helped to mitigate the toxicity of the core CuO NMs only in the long exposure.

3.5 Conclusions

This study highlights some of the critical ecological responses that may be overlooked when conducting acute test with NMs, emphasizing the importance of the evaluation of multiple endpoints to understand the mechanisms of toxicity of NMs, in particular when coating or different dispersants are used to produce SbyD NMs. Results also indicate that examining sublethal effects of NMs can be more realistic and useful to assess toxicity than merely assess lethal effects.

Findings demonstrated that the feeding rate was the most sensitive endpoints when evaluating the chronic ecotoxicity of CuO NMs on *L. stagnalis*, while effects on fecundity and growth appeared to be indirect consequences of the reduction in energy available to the snails due to the arrest or decrease in food consumption. Data suggest that toxicity to snails can occur via food or water borne exposure. Indeed, in this study the high dissolution of the CuO NMs could not be correlated with toxicity.

Regarding the toxicity of SbyD CuO NMs, results were in accordance with the findings from the acute exposure, where the toxicity was attributed to the presence of Cu-phosphate complexes which were likely more bioavailable to snails due to their feeding behaviour. However, to confirm this conclusion, solubility and speciation of the NMs should have been measured more frequently and in the presence of living organisms. In addition, further research is required to assess the accumulation of CuO NMs of *L. stagnalis* after chronic exposure. However, the need of tracer methodologies are required to assess the origin of the accumulated Cu, especially at environmental relevant concentrations, from the high background Cu concentration in *L. stagnalis* which averages 34 ± 26 $\mu g G^{-1}$ (Croteau et al. 2014b).

Chapter 4 Toward the use of long term memory (LTM) formation as a non-invasive endpoint to assess CuO NMs toxicity on the snail, *L. stagnalis*

4.1 Introduction

In the last two decades nanotechnology use has exponentially grown, determining an expansion of its application in various industrial sectors, such engineering, crop protection and pharmaceutical. Alongside, growing concerns have arisen regarding the potential toxicity that products of nanotechnology may pose to human health and the environment due to their enhanced physico-chemical properties, such as size, surface reactivity, compared with their bulk counterpart. Copper oxide nanomaterials (CuO NMs) are one of the NMs with high commercial interest, due, manly, to their biocidal proprieties. They are mostly used as wood preservative agent and antifouling paints leading to a high risk of their wash off in the aquatic environment, where no-target organisms, such as snails, may be affected.

Various studies (Buffet et al. 2011, Pang et al. 2012, Buffet et al. 2013, Pradhan et al. 2015) have demonstrated that behavioural biomarkers are sensitive tools to assess the impact of NMs compared to endpoints representative of metabolic disruption, such as growing, bioaccumulation and reproduction (Buffet et al. 2013). Exposure to sublethal copper NMs has been shown to alter several behaviours in aquatic organisms due the disruption of different sensory systems such as light receptors, mechanoreceptors, chemoreceptors. For instance, in Sovová et al. (2014), a short exposure (12h) to Cu NMs (50 μgL^{-1} Cu) impaired the olfactory mediated behaviours of juvenile rainbow trout (*Oncorhynchus mykiss*) reducing the organism ability to detect alarm substance stimulus in water (Sovová et al. 2014).

Locomotory and feeding behaviour can also be adversely affected by copper NMs exposure. Buffet et al. (2013) in a mesocosm experiment showed that burrowing behaviour was one of the most sensitive endpoint to assess the toxicity of CuO NMs in two endobenthic species, the clam *Scrobicularia plana* and the worm *Hediste diversicolor*. Respectively, after 21 and 14 days of exposure their burrowing kinetics were significantly affected. Furthermore, the feeding rate, after day 14, was significantly reduced in clams exposed to CuO NMs. Feeding behaviour was also affected on the invertebrate shredder *Allogamus ligonifer* exposed to increasing CuO NMs concentrations (0, 25 and 75 mgL^{-1} Cu) and effects were stronger as NMs size decreased (up to 83.3% inhibition for 12 nm particles) (Pradhan et al. 2015). Finally, CuO NMs are also able to alter the respiration behaviour of fish (Villarreal et al. 2014) and bacteria (Wahab et al. 2013), provoking an increase in gill ventilation in the first study and a respiration inhibition in the latter.

Such adverse effects can be correlated with the loss of the organisms of their natural predator avoidance behaviour due to detrimental effects on of their neurological functions which mediate the organism to respond under stress to alarm cues (Turner et al. 1999, O'Gara et al. 2004, Orr et al. 2007, Dalesman and Rundle 2010, Sovová et al. 2014). CuO NMs have been showed to cross the blood-brain barrier (Sharma and Sharma 2007, Al-Bairuty et al. 2013) indicating a potential neurotoxicity of this NM. Indeed, neurotoxicity is defined as "*any adverse effect, permanent or reversible, on the structure or function of the central and/or peripheral nervous system originated by a biological, chemical, or physical agent that diminishes the ability of an organism to survive, reproduce, or adapt to its environment*" (Andrade et al. 2017).

The neurotoxicity of CuO NMs was investigated by An et al. (2012) by exposing mice for 14 days to those NMs. Cognitive abilities were tested using a Morris water maze (MWM) test, one of the most effective behavioural test for examining learning and memory in rodents. Results demonstrated a marked impairment of learning and memory abilities in CuO NMs treated mice. These finding were attributed to the neuronal damage induced by impairing oxidation–antioxidation homeostasis of the hippocampus. Similarly, Bulcke et al. (2017) reviewing the existing literature on the neurotoxicity of copper, concluded that the reported toxicity of CuO NMs to brain cells was most likely mediated by accelerated ROS production and oxidative damage.

In this research study, the potential neurotoxicity of CuO NMs was tested using the model species *L. stagnalis*, which has been widely used as model system able to elucidate neuronal mechanisms investigating how learning and memory are encoded within the nervous system (Orr et al. 2007).

The ability to learn and form memory can enhance snails' fitness by allowing them, for example, to remember predators, food sources or conspecific interactions (Sugai et al. 2006, Orr and Lukowiak 2008, Hughes et al. 2017); however, if under stress their cognitive abilities might be affected. The effects of stressors on memory is best described by the Yerkes-Dodson/Hebb (Y-D/H) Law, which dictates that memory is formed when there is an optimal stress level, but with too much or too little stress memory formation is less than optimal (Young et al. 2017). Indeed, different combinations of stressors have been shown to alter the snails' ability to learn and form memory following operant conditioning of aerial respiratory behaviour (Lukowiak et al. 2014).

Operant conditioning is a learning process in which the occurrence of a specific behaviour is increased or decreased through positive or negative reinforcement each

time the behaviour is exhibited, so that the subject associate the outcome the reinforcement with the behaviour (Skinner 1965) .

In *L. stagnalis* aerial respiration can be operantly conditioned, such that the animal learns not to perform the behaviour, and long-lasting memory can be demonstrated (Lukowiak et al. 2006). This operant conditioning procedure has been widely used in studies of learning and memory since 1996, when Lukowiak et al. exploited the bimodal breathing behaviour of *Lymnaea* to determine their capability for learn and form memory. *L. stagnalis* can perform either skin or lung respiration through a breathing tube known as the pneumostome (Lukowiak et al. 1996). In a hypoxic environment (≤ 5% O_2), the frequency of aerial respiration increases significantly in association with an increase on the respiratory movements, before the snail submerges. Thus, in this study, snails were operantly conditioned to not open their pneumostome in a hypoxic environment, which favours aerial respiration, by applying a gentle tactile stimulus to the pneumostome as it attempted to open (Lukowiak et al. 1996).

In this research study, it was hypothesized that the effect of chronic exposure to CuO NMs on cognitive processes (*e.g.* memory formation) may be an earlier indicator of toxicity compared to the conventional LC50, as shown by Young et al (2017) exposing snails to silver (Ag) NMs. In this instance, authors found that Ag NMs and $AgNO_3$ at relatively low concentrations 10 μgL^{-1} and 5 μgL^{-1} both substances enhance the ability of the snails to form memory. However, when increasing concentration to 50 μgL^{-1} and 10 μgL^{-1}, respectively, for Ag NMs and $AgNO_3$ memory formation was blocked. The authors attributed the memory deficits observed in snails exposed to $AgNO_3$ to induced oxidative stress. In contrast, they suggested that Ag NMs acted as an irritant on a sensory structure such as the osphradium, causing a stressful response similar to other stressors (*e.g.* thermal) which alter the snails' ability to form memory (Young et al. (2017).

4.2 Materials and methods

4.2.1 Experimental design

Young adult of *L. stagnalis* (22 ± 2 mm) were exposed in a semi-static experiment to Cu as CuO NMs and $CuSO_4$ for 30 days in OECD 203 medium, consisting of: 79 mgL^{-1} Ca^{2+}, 38 mgL^{-1} Mg^{2+}, 12 mgL^{-1} Na^{+}, 17 mgL^{-1} and 2 mgL^{-1} K^{+} with a pH of 7.7. General water hardness was 250 mgL^{-1} $CaCO_3$. To allow results homogeneity, shell size length was measured using a digital calliper at the start of the experiment. Four

replicates, of five snails each, were exposed to each concentration and controls in a 1 L glass beaker. Snails were fed *ad libitum* and a 100% medium renewal was performed every 3 days to maintain exposure concentrations over the experiment duration and water quality.

In order to individuate easily the different snails during the training sessions (see 4.2.1.2), snails were individually labelled at least 48 hr prior starting exposure by gluing a small number printed on waterproof paper onto the shell above the position of the pneumostome (Fig. 4-1).

Figure 4-1 Printed number is glued on the snail's shell in proximity of the pneumostome.

As described by Lukowiak and Dalesman (2013), snails were tested in groups of 10 individuals in each beaker. These authors found that 10 was the optimal number to process a large number of snails avoiding contact between individuals which could disrupt breathing patterns. All exposures concentrations were based on mass of copper added; dosing was accomplished by adding the desired aliquot from the stocks into the exposure vessels. In this Chapter, test chemicals used were equivalent to chapter 3, thus characterisation data are not reported here.

4.2.1.1 Respiration behaviour

In well oxygenated water, *L. stagnalis* favour cutaneous respiration being able to obtain adequate gas exchange via the skin, when the water becomes hypoxic aerial respiration is increases significantly. Snails reaching the water-air interface where exhibit several opening and closing movements of their respiratory orifice, the pneumostome, providing an airway between the rudimental lung and the atmosphere. These respiratory movements are often repeated several times before the snail submerges (Lukowiak and Syed 1999).

The snails breathing behaviour was observed free of any stimuli to determine if the treatments significantly alter baseline aerial respiratory behaviour. This is done to

ensure that any memory observed, which is measured as a significant decrease in the number of attempted pneumostome openings, is not simply a chemical inhibition of aerial respiration (Rosenegger et al. 2008). Initial aerial breathing behaviour prior to CuO NMs exposure (reported in figures as "Pre") was assessed in standard medium alone. Subsequent breathing behaviour was then assessed at 10, 20 and the end of the chronic exposure to the NMs (reported in figures as "Post10days, Post20days and Post 30days"). To assess aerial breathing behaviour, in 1 L glass beaker were placed 500 ml of media, either control or containing NMs. This media was then made hypoxic by bubbling N_2 through the water for 20 min to promote snails' aerial respiration. Snails were acclimated to the beaker's hypoxic environment for 10 min, and total breathing time (TBT, time during which the snail keep the pneumostome open) and total breathing numbers (TBN, numbers of successful pneumostome openings) were recorded over a period of 30 min (Fig. 4-3).

4.2.1.2 Long-term memory formation test

Long-term memory (LTM) formation was assessed using operant conditioning of aerial respiration in hypoxia (Lukowiak et al. 1996). Snails collected from the chronic exposure to NMs were tested for LTM formation 72 hours after two training sessions (TS) (TS1-1, TS1-2, TS2-1, TS2-2) lasting 30 min, separated by an hour at 24 and 48 hours (Fig. 4-2). After the medium was made hypoxic, snails were acclimated for 10 min to the new environment and then trained by a negative reinforcement in the form of a gentle tactile stimulus (poke) on the pneumostome each time it attempted to perform aerial respiration, such that the pneumostome closed but the snail did not fully withdraw into its shell (Fig. 4-2). Thus, in control conditions, snails reduce the number of attempted pneumostome openings due to the repeated application of the negative reinforcement.

Figure 4-2 Snail were poked gently on their pneumostome every time they attempted to perform aerial respiration at the water/air surface. Credit: Ken Lukowiak.

This was followed by a memory test (Mt) 24 hours after the second training session on the second day, using identical protocol to a single training session (Byzitter et al. 2012). Long-term memory was determined to be present if the number of attempted pneumostome openings in Mt session was significantly lower than the first training session on day 1 (TS1-1) but not significantly higher than the last training session on day 2 (TS2-2) (Parvez et al. 2006). Occasionally, during the duration of the experiment, snails did not perform aerial respiration, in which case snails were excluded from analysis. This resulted in a final total number of snails different for each treatment, which was sometimes additionally affected by mortality taking place during the 30 days exposure.

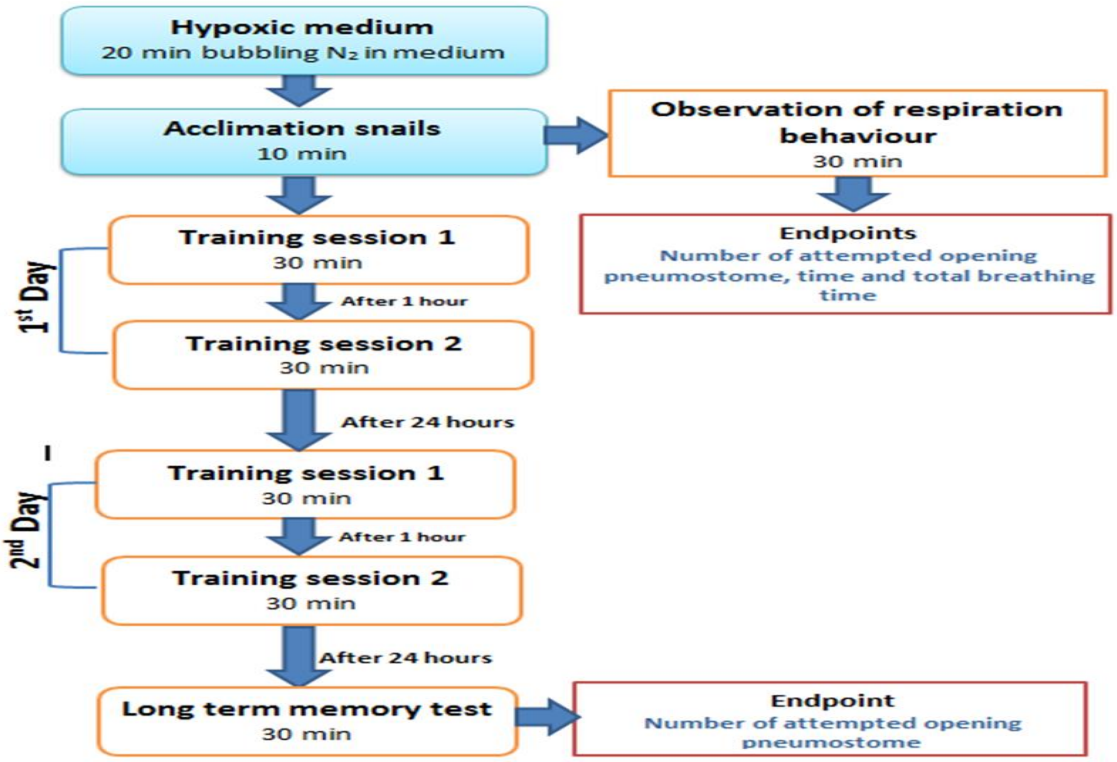

Figure 4-3- Experimental test design for the assessment of the respiration behaviour during 30 days of exposure and LTM formation after 30 days of exposure of young adults of *L. stagnalis*.

4.2.2 Data analyses

To determine whether the treatments influenced the respiration behaviour when compared to the start of the exposure, and whether the number of attempted pneumostome openings was significantly altered as a result of operant conditioning, a repeated-measures one-way ANOVAs tests (RMANOVA) using Prism® version 6.01 statistic programme. All data were checked for homogeneity of variance using Mauchly's test for sphericity in IBM® SPSS Statistics® version 24. If the data did not pass the test ($p < 0.05$) a Geisser-Greenhouse correction was applied to perform RMANOVA. If the ANOVA was significant ($p < 0.05$), a post hoc Tukey multiple comparison test was performed to show significant differences between sessions ($p < 0.05$).

4.3 Results

4.3.1 Respiration behaviour

At the start and during the 30 days of exposure to Cu as CuO NMs or $CuSO_4$, respiration behaviour of the snails was monitored in order to avoid interpretation bias of the LTM formation results.

Initial studies were performed over 30 days to investigate the chronic effect of pristine CuO NMs (H_2O) and $CuSO_4$, as ionic Cu control, to the homeostatic behaviour of young adults (22 ± 2 mm) of the freshwater gastropod *L. stagnalis*, to establish a baseline behavioural response in the two different forms of Cu, ionic and nano. Concentrations tested ($CuSO_4$: 0-60 μgL^{-1} Cu; pristine CuO NMs: 0-150 μgL^{-1} Cu) were chosen up to the lowest concentration that would significantly inhibit the normal respiration of the snails, either in term of total time spent breathing (open pneumostome, TBT) or total number of successful pneumostome openings (TBN).

Overall, respiration behaviour was not altered during the 30 days of exposure for all the unexposed snails (control) (Fig. 4-4), indicating that the experimental method did not interfered with the gathered findings. In contrast, a clear concentration-time response relationship was revealed for exposure to Cu in either ionic or nano form.

Results from the exposure to $CuSO_4$ showed that at the lowest concentration tested of 20 μgL^{-1} Cu snails were affected by the exposure to Cu. A slightly, although no significant, decrease on the breathing time and number of pneumostome opening was revealed after 10 and 20 days of exposure (Fig. 4-4 and 4-5). At the end of the experiment, a significant decreased in the number of successful pneumostome openings was recorded compared with the start of the experiment (RMANOVA, $F_{(3,27)} = 3.58$; $p = 0.03$). Multiple comparison post-hoc Tukey test revealed that at 40 μgL^{-1} Cu a significant decrease was observed in both TBT and TBN after 30 days of exposure. Finally, at the highest concentration tested (60 μgL^{-1} Cu), snails reduced severely and significantly their respiration activity, so much so that after 30 days of exposure snails were not responding to the hypoxic environment in which the test were performed (RMANOVA, TBT: $F_{(1.13,10.22)} = 18.96$; $p = 0.001$; TBN: $F_{(1.14,10.30)} = 22.94$; $p = 0.0005$) (Fig. 4-5 and 4-5).

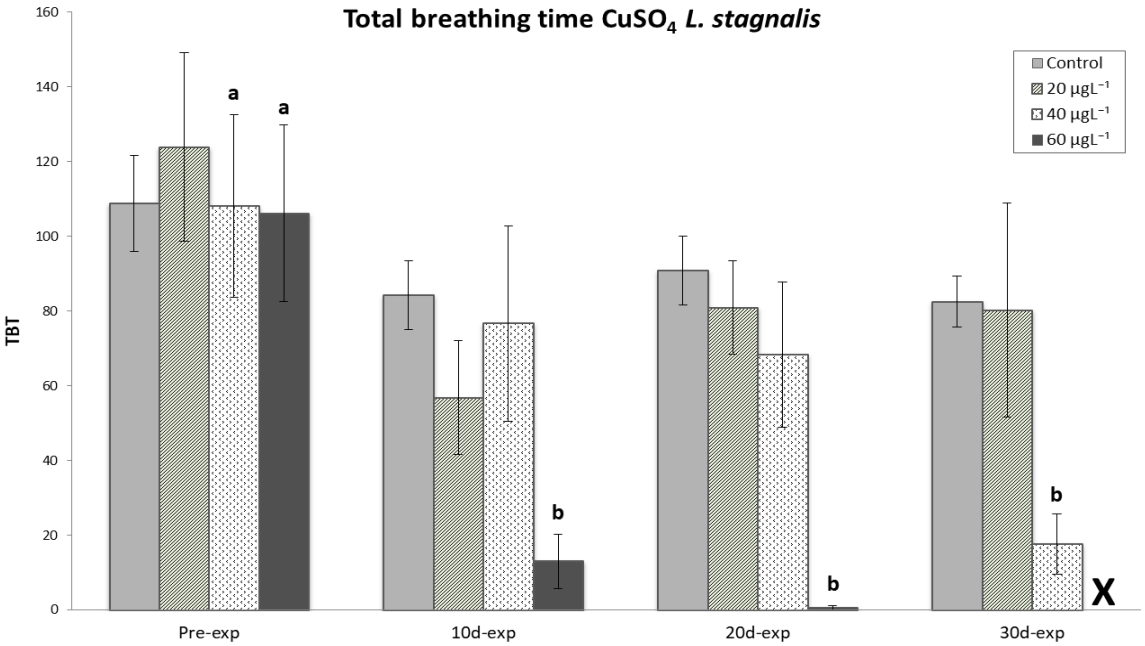

Figure 4-4 Total means of breathing time (opened pneumostome) over 30 min at increasing Cu concentrations as $CuSO_4$ before exposure (pre) and after 10, 20 and 30 days of exposure. X was drawn when snails were not responding to the hypoxic environment in which the test was performed (n = 10, error bars are SEM). Different letters indicate significance between the different time points within the same exposure concentration ($p < 0.05$).

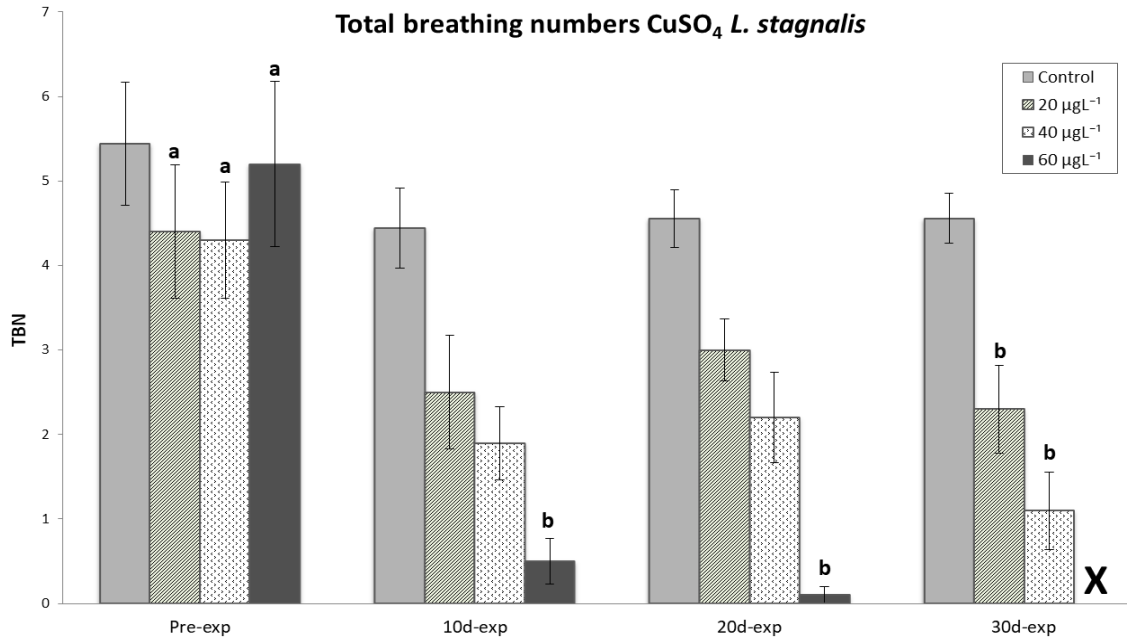

Figure 4-5 Total means of number of times of successful pneumostome openings over 30 min at increasing Cu concentration as $CuSO_4$ before exposure (pre) and after 10, 20 and 30 days of exposure. X was drawn when snails were not responding to the hypoxic environment in which the test was performed (n = 10, error bars are SEM). Different letters indicate significance between the different time points within the same exposure concentration ($p < 0.05$).

Findings gathered from the chronic experiment performed exposing snails to increasing concentrations of pristine CuO NMs, revealed an adverse effect of the NM at concentration 4 times lower than the $LC50_{30d}$ found on the previous experiment (see Chapter 3, Fig. 3-2). At 100 μgL^{-1} Cu, up to 10 days of exposure snails exhibited a normal respiration behaviour, which was significantly different at the end of the

experiment for both endpoint investigated (RMANOVA, TBT: $F_{(3,24)} = 7.19$; $p = 0.001$; TBN: $F_{(3,24)} = 7.50$; $p = 0.001$). However, data showed that at this concentration, after 20 days of exposure, the capability to crawl to the air/water interface to perform aerial respiration was firstly affected (Fig. 4-7); consequently, snails spent more time with the pneumostome opened to allow gas exchange (Fig. 4-6). Finally, at 250 µgL^{-1} Cu, snails were significantly affected by the exposure already after 10 days compared with the start of the experiment.

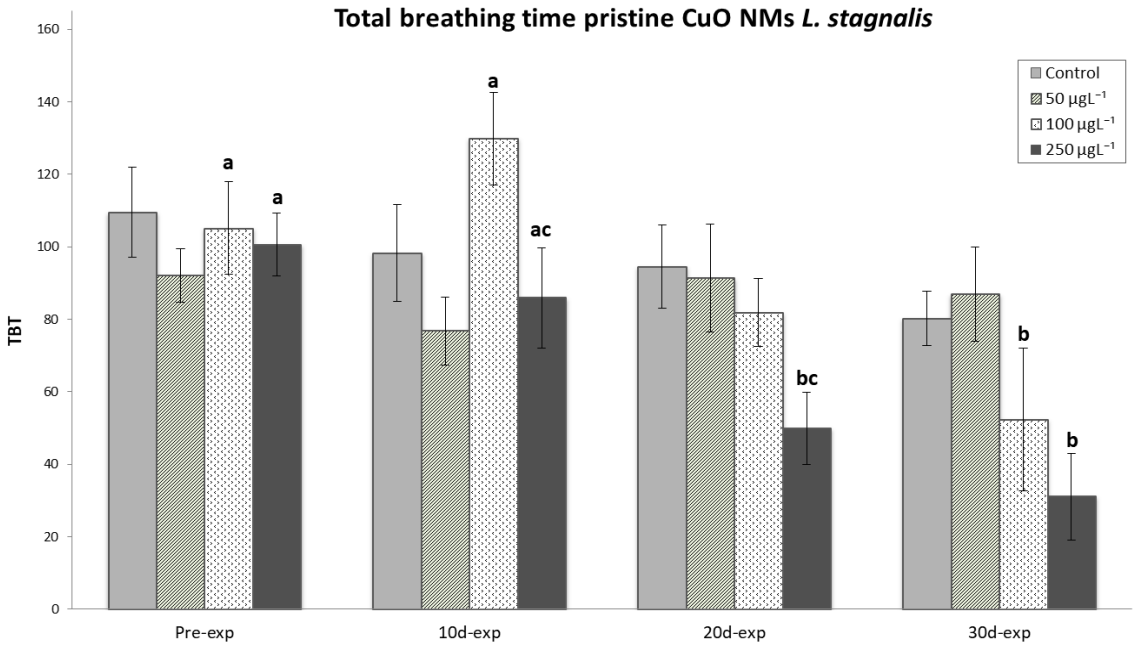

Figure 4-6 Total means of breathing time (opened pneumostome) over 30 min at increasing Cu concentrations as pristine CuO NMs, before exposure (pre) and after 10, 20 and 30 days of exposure (n = 10 for control and 250 µgL^{-1} Cu; n = 9 for 50 and 100 µgL^{-1} Cu, error bars are SEM). Different letters indicate significance between the different time points within the same exposure concentration ($p < 0.05$).

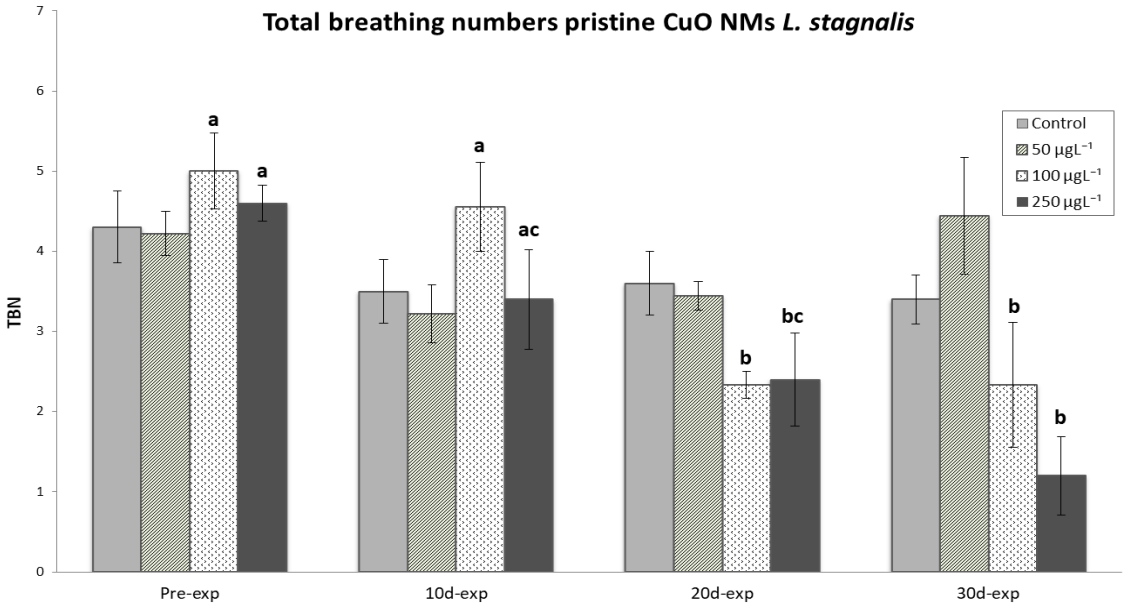

Figure 4-7 Total means of number of times of successful pneumostome openings over 30 min at increasing Cu concentrations as pristine CuO NMs, before exposure (pre) and after 10, 20 and 30 days of exposure (n = 10 for control and 250 µgL^{-1} Cu; n = 9 for 50 and 100 µgL^{-1} Cu, error bars are SEM). Different letters indicate significance between the different time points within the same exposure concentration ($p < 0.05$).

Experiments using the SbyD CuO NMs started by investigating the toxicity of pristine CuO NMs functionalised in phosphate buffer (PBS), CuO(PO_4^{3-}) NMs.

Results showed that PBS had an additive effect on the toxicity of the pristine CuO NMs. At the highest concentration tested, 200 µgL^{-1} Cu, after 20 days of exposure snails were not responding to the hypoxic environment (Fig. 4-8 and 4-9), making it impossible to evaluate any of the respiration endpoints. In fact, at 100 µgL^{-1} Cu, snails were significantly affected by the exposure to CuO(PO_4^{3-}) NMs, for both endpoints after 10 days of exposure (RMANOVA, TBT: $F_{(3,24)} = 13.01$; $p < 0.0001$; TBN: $F_{(3,24)} = 9.38$; $p = 0.0003$). In contrast, at 50 µgL^{-1} Cu, although the mean time spent to perform aerial respiration along the 30 days of exposure was not affected (Fig. 4-8), snails showed a significant decrease in the numbers of successful pneumostome openings after 20 days of exposure (RMANOVA, $F_{(3,24)} = 5.69$; $p = 0.004$).

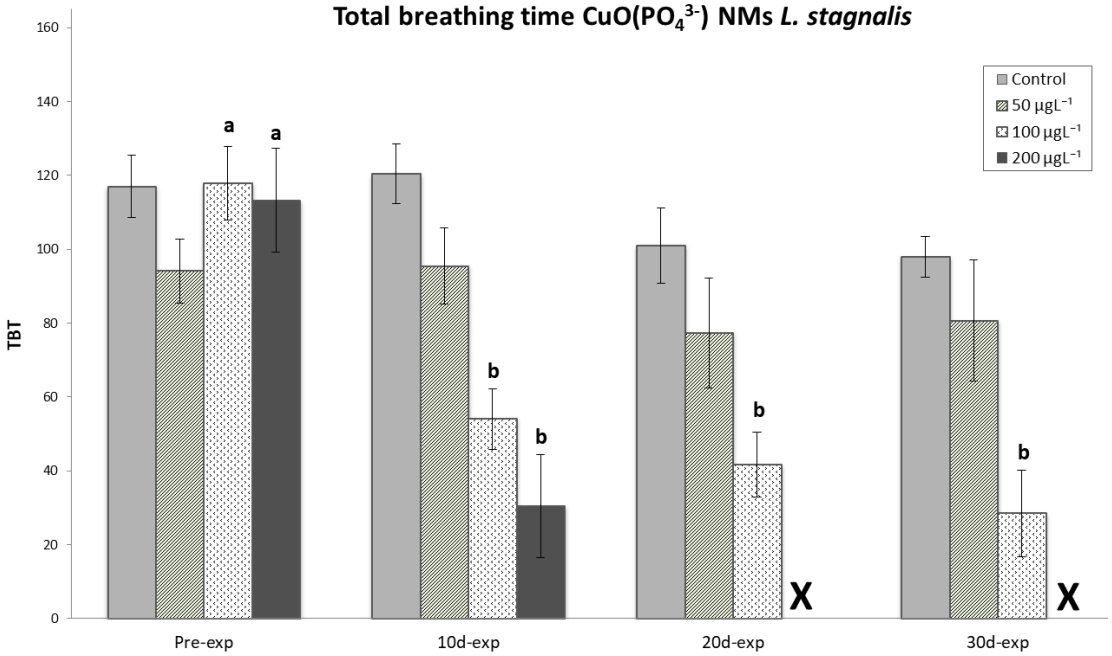

Figure 4-8 Total means of breathing time (opened pneumostome) over 30 min at increasing concentration of Cu as pristine CuO NMs functionalised in phosphate buffer, before exposure (pre) and after 10, 20 and 30 days of exposure. X was drawn when snails were not responding to the hypoxic environment in which the test was performed (n = 20 for control; n = 9 for 50, 100 and 200 µgL^{-1} Cu, error bars are SEM). Different letters indicate significance between the different time points within the same exposure concentration ($p < 0.05$).

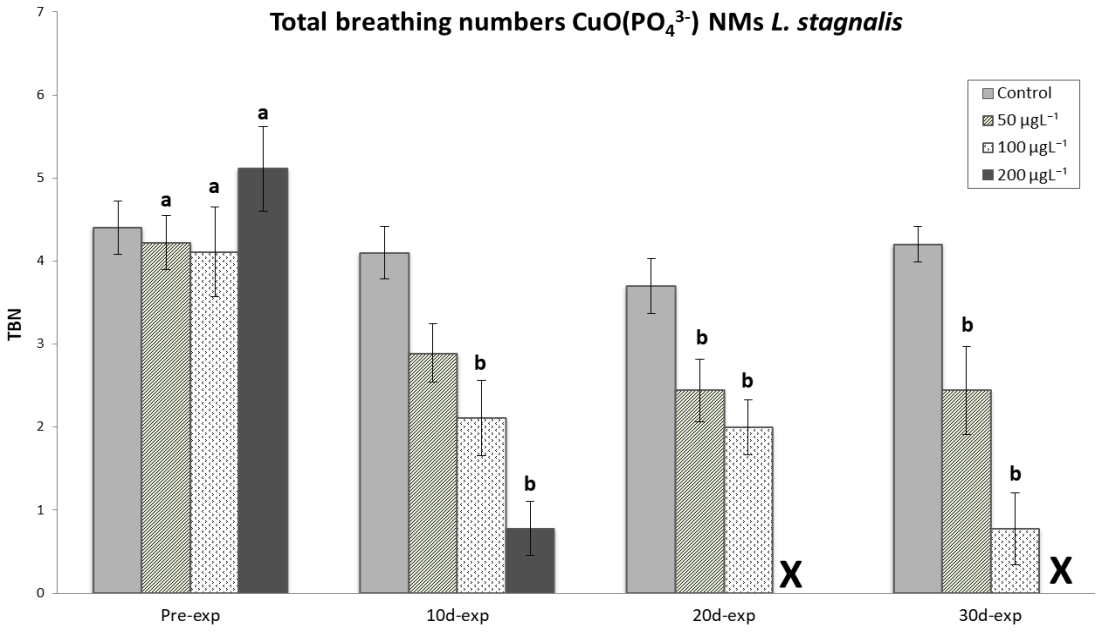

Figure 4-9 Total means of number of time of successful pneumostome openings over 30 min at increasing concentration of Cu as pristine CuO NMs functionalised in phosphate buffer, before exposure (pre) and after 10, 20 and 30 days of exposure. X was drawn when snails were not responding to the hypoxic environment in which the test was performed (n = 20 for control; n = 9 for 50, 100 and 200 µgL^{-1} Cu, error bars are SEM). Different letters indicate significance between the different time points within the same exposure concentration ($p < 0.05$).

Results from the experiments performed using CuO NMs coated with polyvinylpyrrolidone (PVP) are, overall, in accord with the chronic lethality data (Chapter 3, Fig. 3-3). Indeed, CuO-PVP(PO_4^{3-}) NMs and CuO-PVP(H_2O) NMs

induced, respectively, the highest and the lowest toxicity among all the CuO NMs investigated.

Results, from experiments performed with CuO-PVP(PO_4^{3-}) NMs, were not analysed for the concentration of 200 μgL^{-1} Cu due to the high mortality (80%) recorded. Data showed a significant inhibition of the respiration behaviour at the lowest concentration tested of 50 μgL^{-1} Cu after 30 days of exposure (RMANOVA, TBT: $F_{(3,21)} = 9.06$; $p = 0.0005$; TBN: $F_{(3,24)} = 9.38$; $p = 0.0003$). In contrast, at 100 μgL^{-1} Cu snails were affected by the exposure to CuO-PVP(PO_4^{3-}) NMs after 10 days; at 30 days snails were rarely crawling to the surface to perform aerial respiration (RMANOVA, TBT: $F_{(3,21)} = 15.47$; $p < 0.0001$; TBN: $F_{(1.94,13.59)} = 38.84$; $p < 0.0001$) (Fig. 4-10 (1 and 3)).

In contrast, snails overall were poorly affected by the exposure to CuO-PVP NMs functionalised in Milli-Q water. Indeed, multiple comparison Tukey test revealed a significant decrease on the respiration activity only for snails exposed to 200 μgL^{-1} Cu of CuO-PVP(H_2O) NMs after 20 and 30 days of exposure compared with the measurement taken before start of the exposure (RMANOVA, TBT: $F_{(3,18)} = 24.61$; $p < 0.0001$; TBN: $F_{(3,18)} = 6.1$; $p = 0.005$) (Fig. 4-10 (2 and 4)).

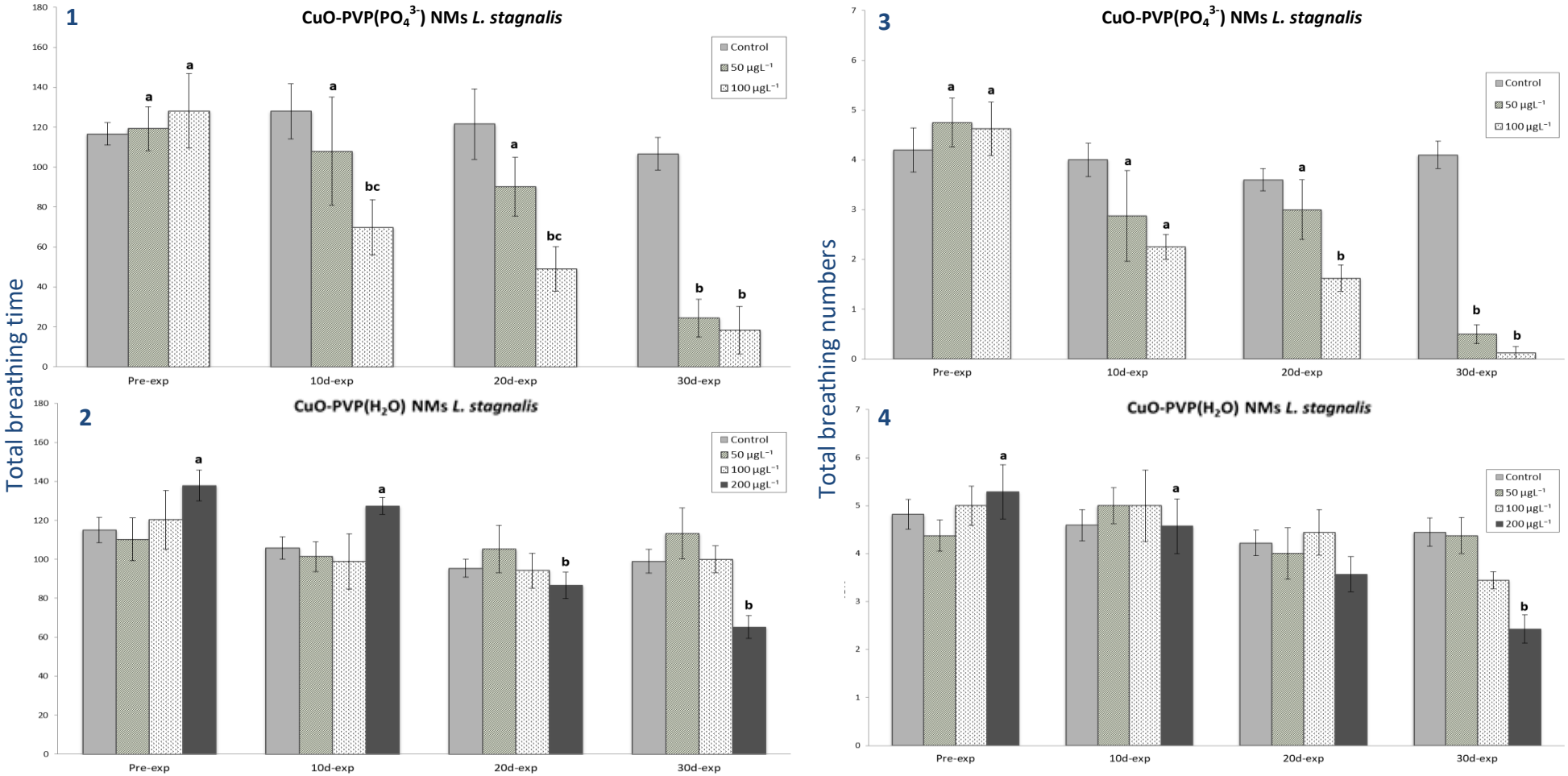

Figure 4-10 Respiration behaviour of snails exposed to CuO NMs coated with PVP. 1 and 2) Total means of breathing time (opened pneumostome) over 30 min at increasing concentration of Cu as CuO-PVP NMs functionalised in PBS (1) and Milli-Q water (2); 3 and 4) Total means of number of time of successful pneumostome openings over 30 min at increasing concentrations of Cu as CuO-PVP NMs functionalised in PBS (3) and Milli-Q water (4). Different letters indicate significance between the different time points within the same exposure concentration ($p < 0.05$).

Experiments carried out exposing the snails to CuO NMs coated with ascorbate (ASC) showed a toxicity trend (Fig. 4-5) different from the other two CuO NMs functionalised in PBS, CuO(PO_4^{3-}) NMs and CuO-PVP(PO_4^{3-}) NMs (Fig. 4-8, 4-9 and 4-10).

Overall, multiple comparison post hoc Tukey test revealed that the respiration behaviour was not affected when snails were exposed for 30 days to 50 μgL^{-1} Cu of CuO-ASC NMs functionalised in either medium (PBS or Milli-Q water). In contrast, at the highest concentration tested of 200 μgL^{-1} Cu, a significant inhibition of the respiration endpoints at 30 days of exposure was observed for either functionalisation medium, PBS (RMANOVA, TBT: $Fs_{(3, 24)} = 31.92$ (TBT)/ 21.57 (TBN); $p < 0.0001$) or Milli-Q water (RMANOVA, TBT: $F_{(3,18)} = 24.61$; $p < 0.0001$; TBN: $F_{(1.473,\ 11.79)} = 18.86$; $p = 0.0004$) (Fig. 4-11). Furthermore, it is worth note, that after 30 days, snails exposed to CuO-ASC(H_2O) NMs were more severely affected compared with the CuO-ASC(PO_4^{3-}) NMs, so much so that only 2 snails over the 9 tested were able to crawl up to the air/water surface to perform aerial respiration.

This toxicity trend was also observed at the exposure concentration of 100 μgL^{-1} Cu. Indeed, and interestingly, CuO-ASC(PO_4^{3-}) NMs were shown to be less toxic (Fig. 4-11 (1 and 3)) than the same materials functionalized in Milli-Q water. Snails exposed to CuO-ASC(H_2O) NMs showed a significantly decrease in the time spent performing aerial respiration after 20 days of exposure to 100 μgL^{-1} Cu (RMANOVA, $F_{(3,24)} = 5.37$; $p = 0.006$) (Fig. 4-11 (2)).

In contrast, at the same concentration of 100 μgL^{-1} Cu, no significant effect was revealed in either endpoint investigated, for snails exposed to CuO-ASC functionalised in PBS (RMANOVA, TBT: $F_{(3,18)} = 24.61$; $p < 0.0001$; TBN: $F_{(3,18)} = 6.1$; $p = 0.005$).

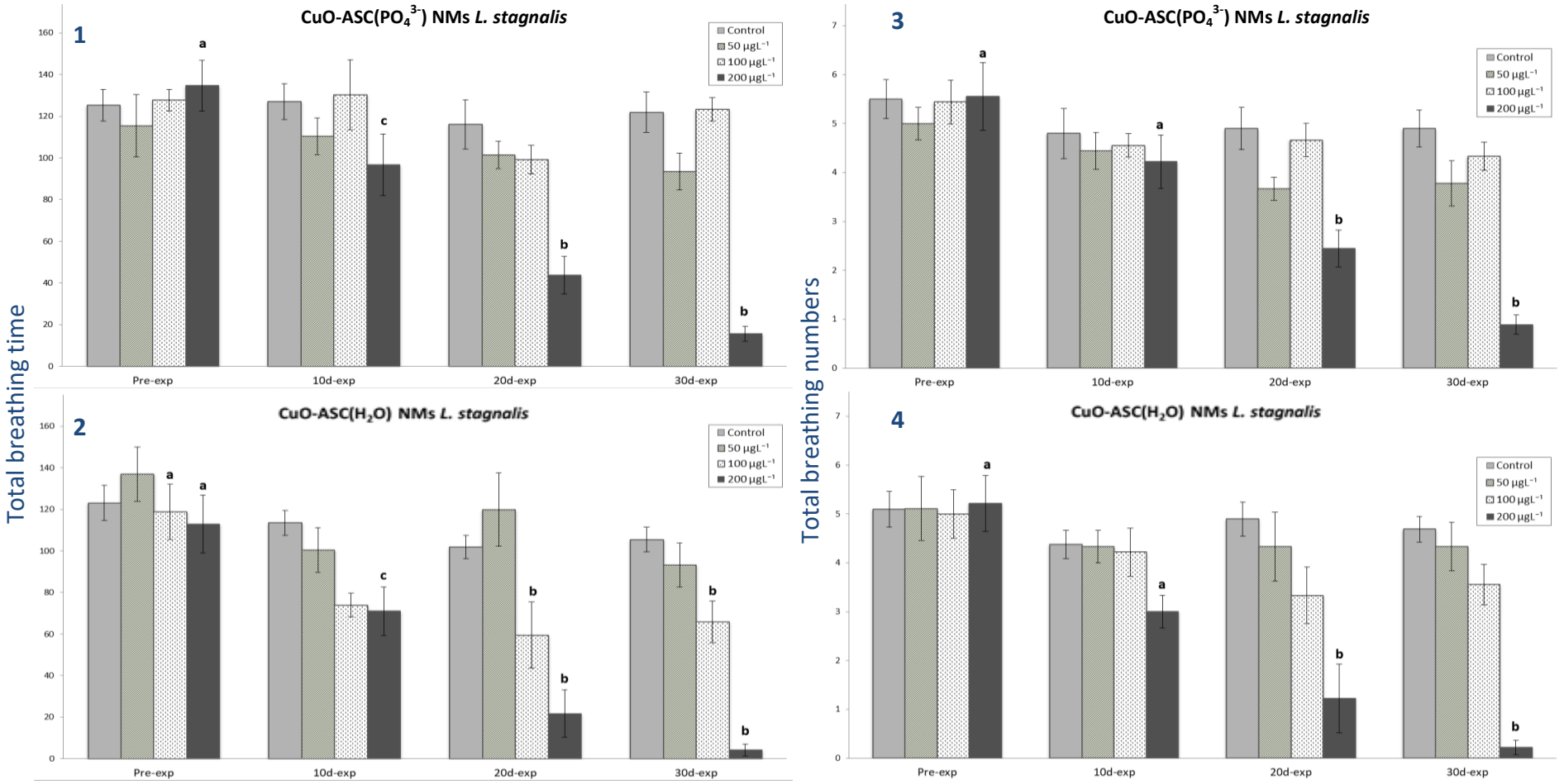

Figure 4-11 Respiration behaviour of snails exposed to CuO NMs coated with ASC. 1 and 2) Total means of breathing time (opened pneumostome) over 30 min at increasing concentration of Cu as CuO-ASC NMs functionalised in PBS (1) and Milli-Q water (2); 3 and 4) Total means of number of time of successful pneumostome openings over 30 min at increasing concentration of Cu as CuO-ASC NMs functionalised in PBS (3) and Milli-Q water (4). Different letters indicate significance between the different time points within the same exposure concentration ($p < 0.05$).

4.3.2 LTM formation test

It was hypothesised that the LTM formation test could be a more sensitive endpoint to detect detrimental effect of NMs than conventional endpoints. Indeed, findings gathered from this research study revealed that by evaluating the cognitive ability of the snails it is possible to detect adverse effects of the exposure to Cu as $CuSO_4$ and CuO NMs at lower concentrations than the conventional L/EC50 values. Overall, data collected from the test performed on the control organisms showed the ability of the snails to learn. Indeed, the number of attempted pneumostome openings obtained during the Mt was significantly lower than obtained at the first training session on day one, TS1-1, but not significantly higher than obtained at the last training session, TS2-2. Furthermore, there was a significant difference in the numbers of the attempted pneumostome openings between the first session at the second day of training, TS2-1, and the Mt session, indicating that although snails showed a significant lower number of attempts at the Mt session, memory was not consolidated (Fig. 4-12, 4-13, 4-14, 4-15, 4-16 and 4-17). Furthermore, results revealed that the occurrence of normal respiration behaviour was essential for snails to be able to learn and then form associative memory.

LTM tests performed using $CuSO_4$, were not performed at the concentration of 60 μgL^{-1} Cu due to the inability of the snails to respond, at the end of 30 days of exposure, to the hypoxic environment in which the test was performed, as showed by the respiration behaviour data (Fig. 4-4 and 4-5).

Results revealed that, interestingly, at both exposure concentrations tested of 20 and 40 μgL^{-1} Cu of $CuSO_4$, snails were able to learn and remember to reduce aerial respiration after the first day of training (day 1); however, it appeared that they could not retain the learned behaviour for more than 24 hrs, probably due to the additional stress imposed by the application of the continuous negative stimulus. Thus, at the third day of test, long-term memory was not formed (Fig. 4-12).

Indeed, one-way repeated-measures ANOVA showed that at 20 and 40 μgL^{-1} Cu of $CuSO_4$ a significant difference on the numbers of attempted pneumostome openings recorded at the two-training session at day 2 compared with the first training session (RMANOVA, 20 μgL^{-1} Cu: $F_{(4, 32)} = 5.647$, $p = 0.002$; 40 μgL^{-1} Cu: $F_{(4, 28)} = 4.63$, $p = 0.005$) (Fig. 4-12).

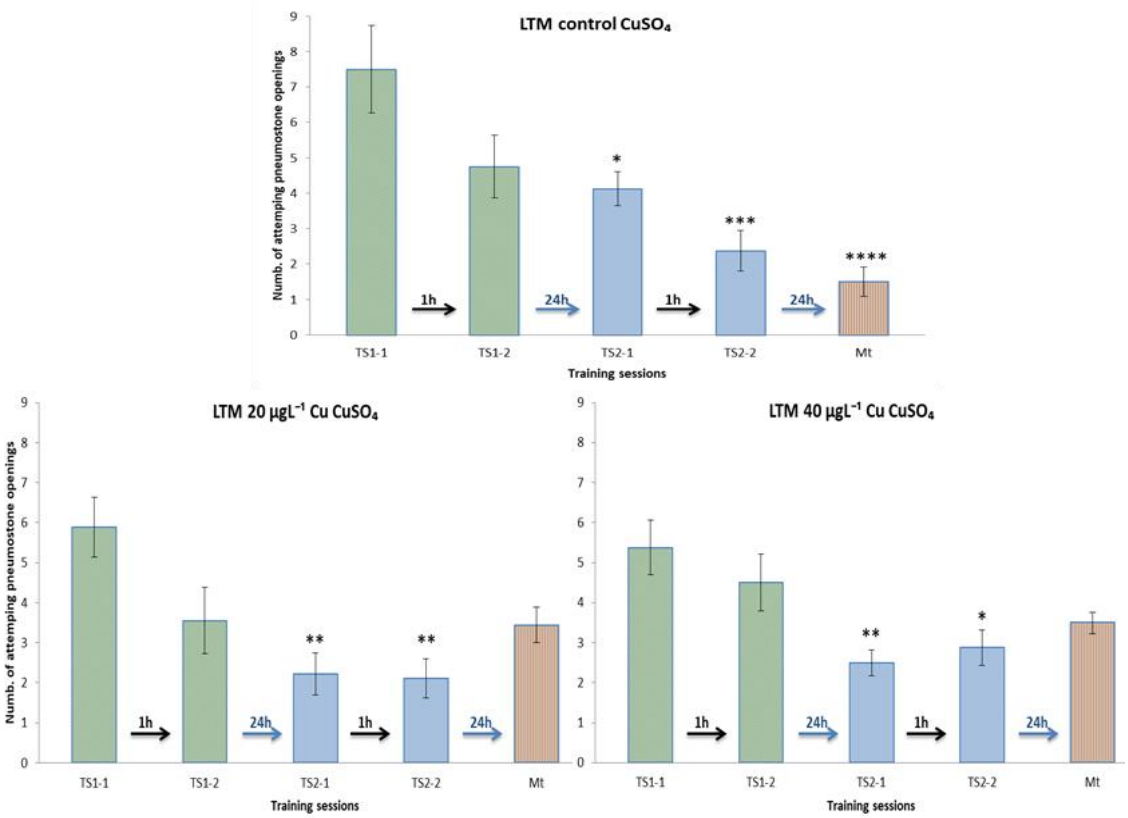

Figure 4-12 Snails were capable of being operantly conditioned. Operant conditioning training of unexposed snails resulted in significantly fewer breathing attempts as training progressed. Exposed snails, to 20 and 40 µgL^{-1} Cu of $CuSO_4$ for 30 days, were instead not able to retain memory of the learned behaviour after the second day of training, resulting in a non-significant difference in the number off attempting pneumostome opening at the MT session. (n = 8 for control and 20 µgL^{-1} Cu, n = 8 for 40 µgL^{-1} Cu; error bars are SEM). Asterisks indicate significant differences compared with the control: * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

LTM experiments could not be performed for snails exposed to 250 µgL^{-1} Cu of pristine CuO NMs due to the severe impairment of the respiration behaviour (Fig. 4-6 and 4-7). Surely, during the first training session, only 3 snails over 10 were responsive to the hypoxic environment (data no reported); therefore, the experiment did not progress.

Overall, data gathered from tests performed using snails chronically exposed to pristine CuO NMs revealed that concentrations exceeding 100 µgL^{-1} Cu interfere with the snails' ability to form memory that lasted more than 24 hours (Fig. 4-13).

Indeed, results of the one-way repeated-measures ANOVA showed that, at 50 µgL^{-1} Cu, there was a significant main effect of the training procedure on the average number of attempted pneumostome opening ($F_{(2.667,\ 21.34)} = 15.80$, $p < 0.0001$). Tukey post hoc test showed that snails learnt and remembered to not perform aerial respiration at the second day of training and to retain the memory during the memory test (Mt) session, indicating that in this case the snails were not negatively affected by the continuous poking.

At 100 µgL^{-1} Cu, repeated measures ANOVA revealed that there was a significant reduction in the means of the number of attempted pneumostome openings ($F_{(3,\ 24)} = 7.50$,

p = 0.001), however multiple comparison showed a non-significant difference of Mt session from the first training session, TS1-1, at day 1 (Fig. 4-13). At the highest concentration tested of 150 ugL$^{-1,}$ the memory test (Mt) was not significantly different from any of the training sessions (Fig. 4-13). This evidence supports the hypothesis that exposure to CuO NMs affects snails' ability to remember at exposure concentrations lower than those do provoked adverse effect on other biological functions.

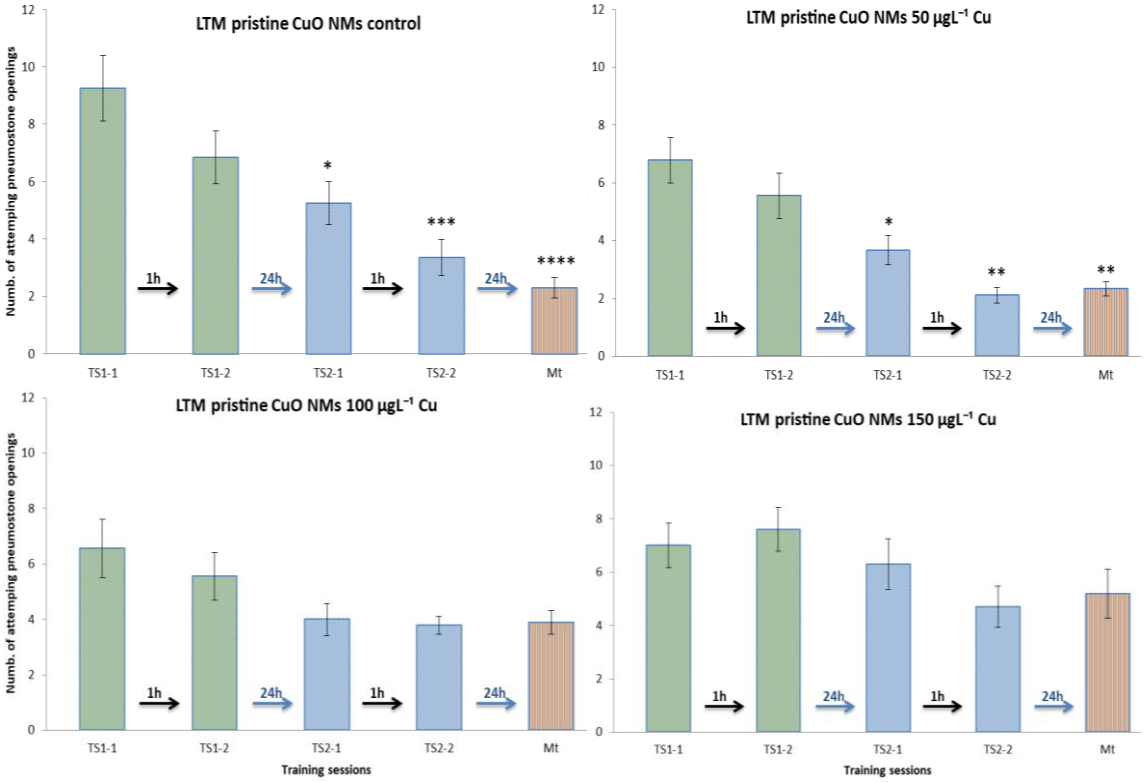

Figure 4-13 Snails were capable of being operantly conditioned. Operant conditioning training, of unexposed snails and exposed snails to 50 µgL^{-1} Cu of pristine CuO NMs, resulted in significantly fewer breathing attempts as training progressed. Exposed snails to 100 and 150 µgL^{-1} Cu for 30 days were instead not able to form memory, resulting in a non-significant difference in the number off attempting pneumostome opening at the Mt session. (n = 20 for control, n = 9 for 50 µgL^{-1} Cu and 100 µgL^{-1} Cu, n = 10 for 150 µgL^{-1} Cu; error bars are SEM). Asterisks indicate significant differences compared with the control: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$.

Data gathered from the exposure studies to SbyD CuO NMs were in accordance with the respiration behaviour toxicity studies. Indeed, exposure to NMs functionalised in PBS resulted in higher toxicity compared to the same material in Milli-Q water, except for ASC, where although data between the two functionalisations media are similar, a slight higher toxicity is observed when Milli-Q water is used as functionalisation medium.

When the effect on learning and memory of pristine CuO NMs functionalised in PBS was assessed, it was highlighted how the presence of PBS resulted in an increase in toxicity of pristine CuO NMs. LTM tests could not be performed at the two highest concentration tested, 100 and 200 µgL^{-1} Cu of CuO(PO_4^{3-}) NMs, due to the strong effect

on the respiration behaviour observed at the end of 30 days of exposure (Fig. 4-8 and 4-9). Snails' cognitive abilities were impaired at the lowest concentration tested of 50 μgL^{-1} Cu. As the training sessions progressed, snails appeared to learn the reinforced negative stimulus given in the first day of training; however, after 24h they were not able to overcome the additional stress due to the application of the negative stimulus, resulting on the loss of memory at the 3rd day (Fig. 4-14).

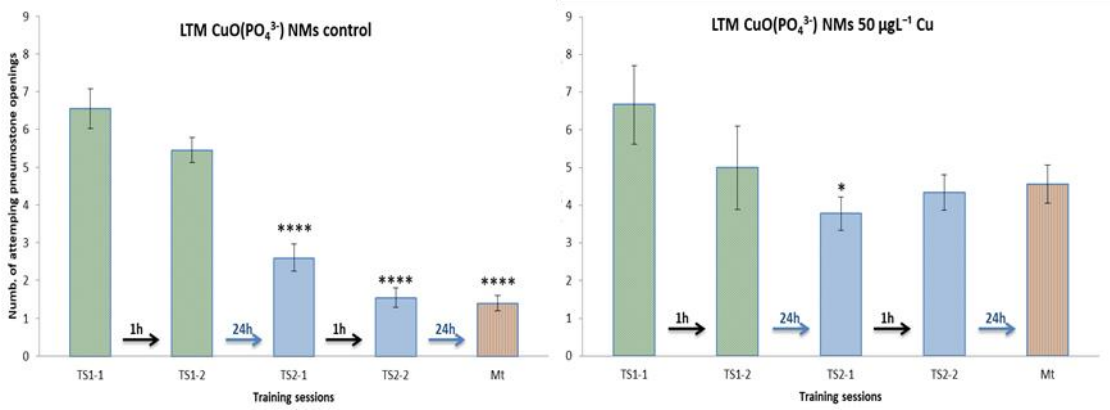

Figure 4-14 Snails were capable of being operantly conditioned. Operant conditioning training, of unexposed snails resulted in significantly fewer breathing attempts as training progressed. Exposed snails to 50 μgL^{-1} Cu of CuO(PO_4^{3-}) NMs, for 30 days, were instead not able to retain memory of the learned behaviour after the second day of training, resulting in a not significant difference in the number off attempting pneumostome opening at the Mt session (n = 20 for control, n = 9 for 50 μgL^{-1} Cu, error bars are SEM). Asterisks indicate significant differences compared with the control: * $p \leq 0.05$ and **** $p \leq 0.0001$.

The additional toxicity afforded by the PBS is further highlighted when snails were exposed to CuO-PVP(PO_4^{3-}) NMs. Here, at all the concentrations tested, 50, 100 and 200 μgL^{-1} Cu, snails were not performing any aerial respiration during the 30 minutes of the test (data not reported); therefore, it was not possible to train the snails to form associative memory.

Contrasting results were, instead, obtained when snails where exposed to the CuO-PVP functionalised in Milli-Q water. CuO-PVP(H_2O) NMs were the least toxic CuO NMs. Although a slight, but not significant, decrease on the number of attempted pneumostome openings was observed with the increase of exposure concentration, snails were able to learn and form memory up to the intermediate concentration tested of 100 μgL^{-1} Cu (RMANOVA, 50 μgL^{-1} Cu: $F_{(4, 24)} = 5.24$, $p = 0.004$; 100 μgL^{-1} Cu: $F_{(4, 28)} = 3.12$, $p = 0.031$) (Fig. 4-15). At 200 μgL^{-1} Cu, due to the small alteration in the respiration behaviour (Fig. 4-10 (3 and 4)), snails formed memory at the second day of training, although they appeared to not be able to retain it for more than 24 h (Fig. 4-15).

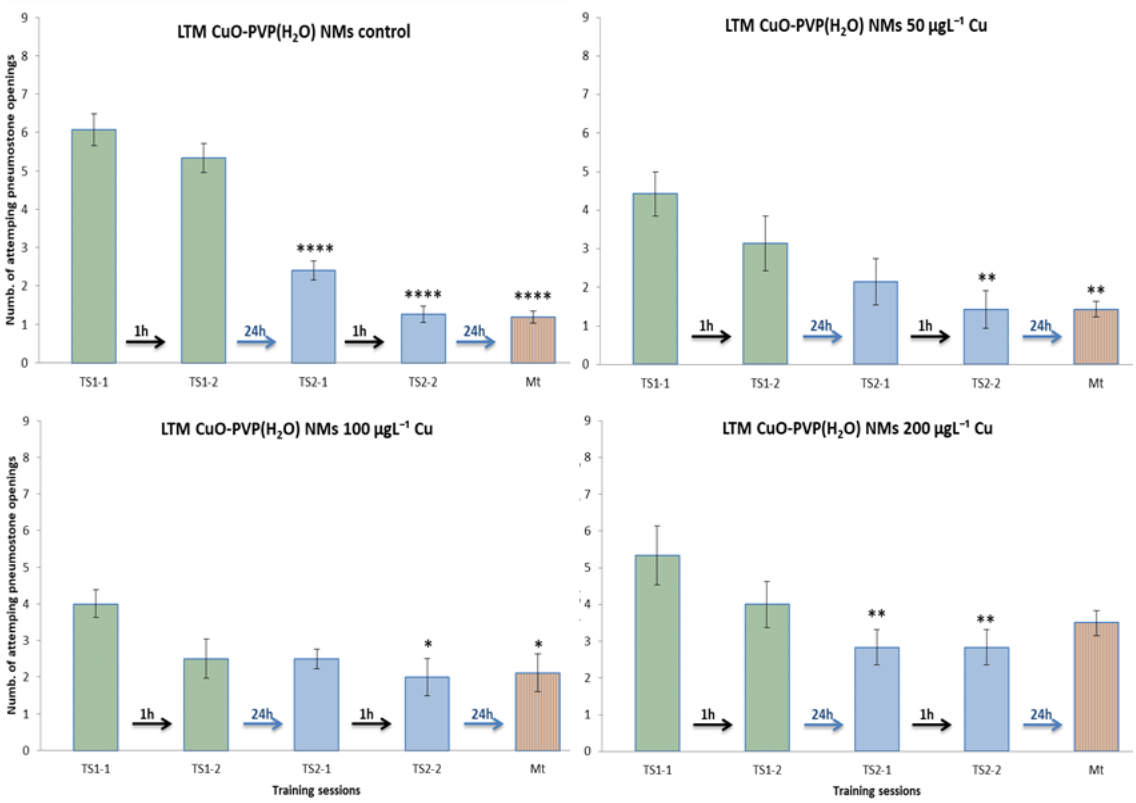

Figure 4-15 Operant conditioning training, of unexposed snails and exposed snails to 50 and 100 µgL^{-1} Cu of CuO-PVP(H_2O) NMs resulted in significantly fewer breathing attempts as training progressed. Exposed snails 200 µgL^{-1} Cu were, instead, not able to retain memory of the learned behaviour after the second day of training, resulting in a non-significant difference in the number off attempting pneumostome opening at the MT session (n = 27 for control, n = 7 for 50 µgL^{-1} Cu, n = 8 for 100 µgL^{-1} Cu and n = 6 for 200 µgL^{-1} Cu; error bars are SEM). Asterisks indicate significant differences compared with the control: * $p \leq 0.05$, ** $p \leq 0.01$, and **** $p \leq 0.0001$.

Finally, findings of the experiment using CuO-ASC NMs revealed, interestingly, a similar response with either of the functionalisations medium used (Fig. 4-16 and 4-17). Indeed, after chronic exposure to either compounds, CuO-ASC(PO_4^{3-}) NMs and CuO-ASC(H_2O) NMs, snails were able to learn and form memory at 50 µgL^{-1} Cu (RMANOVA, CuO-ASC(PO_4^{3-}) NMs: $F_{(4, 32)} = 7.59$, $p = 0.0002$; CuO-ASC(H_2O) NMs: $F_{(4, 24)} = 5.24$, $p = 0.003$) (Fig. 4-16 and 4-17) and they were severely affected by the exposure at 200 µgL^{-1} Cu resulting in failure to perform aerial respiration during the 30 minutes exposure test (data no reported).

Data gathered from snails exposed to 100 µgL^{-1} Cu of either CuO-ASC NMs, confirmed the slight higher toxicity of the CuO-ASC(H_2O) NMs compared with CuO-ASC(PO_4^{3-}) NMs. Tests performed using the latter, demonstrated that snails were able to learn and form memory as the training procedure progressed (RMANOVA, $F_{(4, 28)} = 7.17$, $p = 0.0004$). In contrast, data obtained suggested that snails were more affected by the exposure to CuO-ASC(H_2O) NMs failing to form long term memory at the last test session (Fig. 4-17).

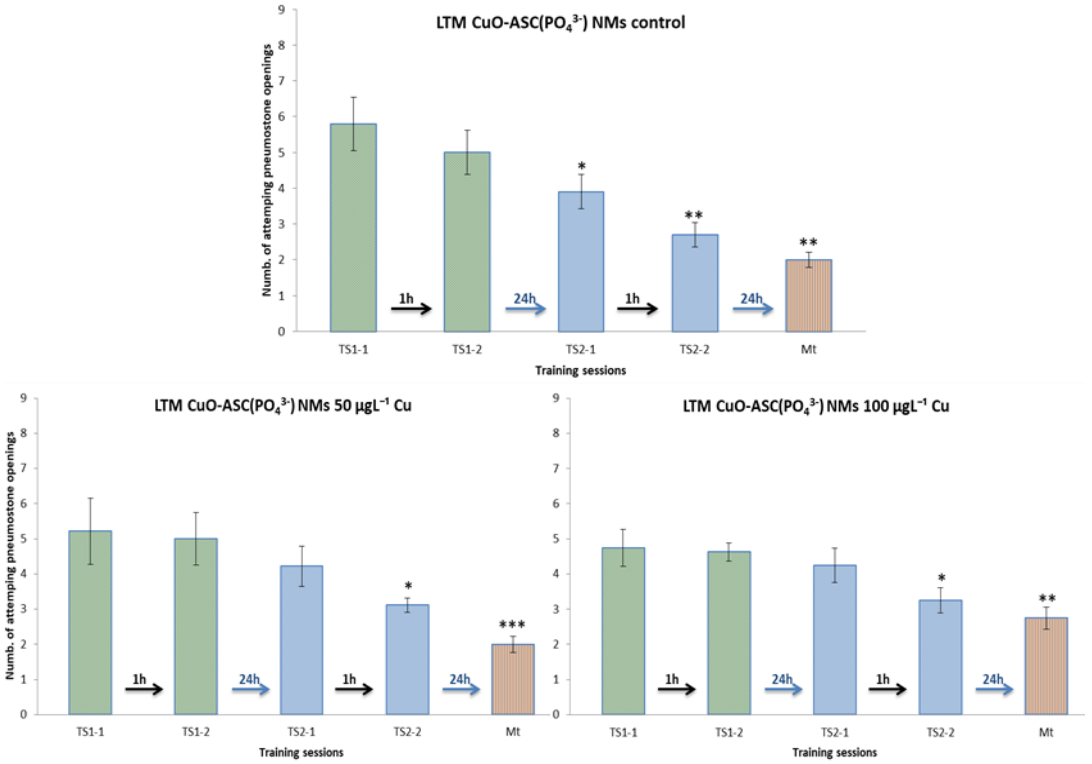

Figure 4-16 Operant conditioning training, of unexposed snails and exposed snails to 50 and 100 µgL^{-1} Cu of CuO-ASC(PO_4^{3-}) NMs resulted in significantly fewer breathing attempts as training progressed (n = 10 for control, n = 9 for 50 µgL^{-1} Cu and n = 8 for 100 µgL^{-1} Cu; error bars are SEM). Asterisks indicate significant differences compared with the control: * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$.

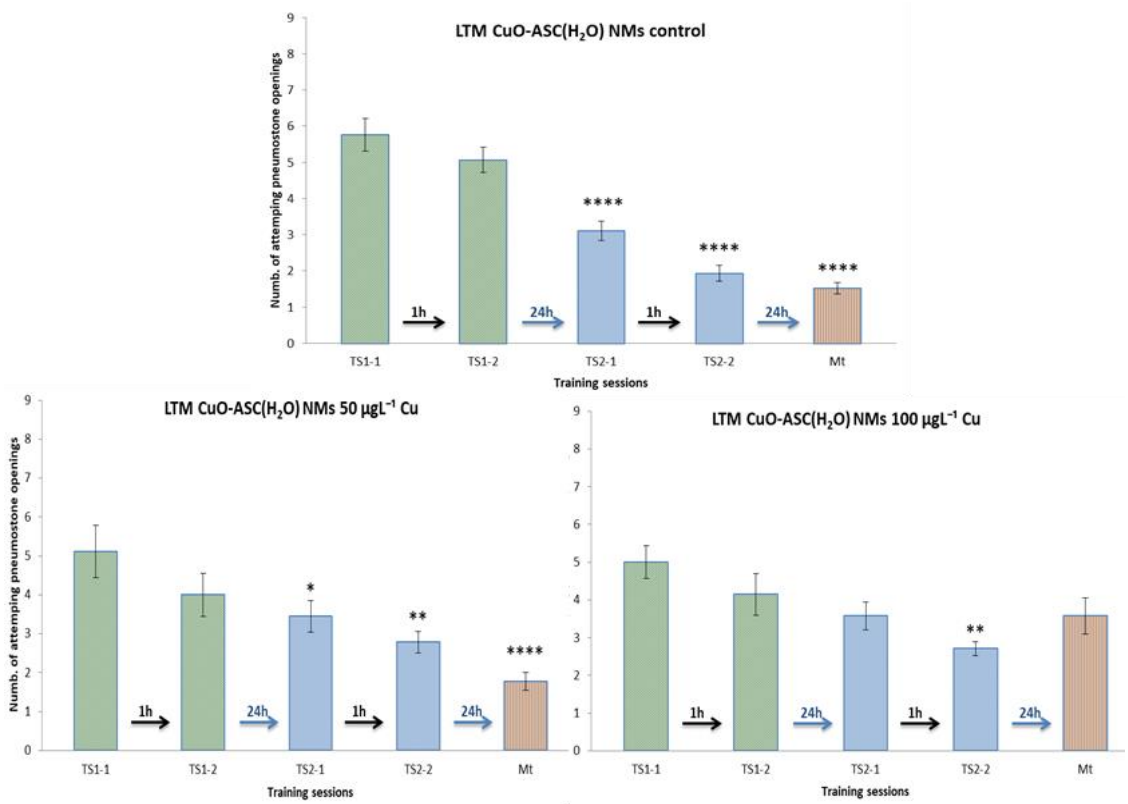

Figure 4-17 Operant conditioning training, of unexposed snails and exposed snails to 50 µgL^{-1} Cu of CuO-ASC(H_2O) NMs, resulted in significantly fewer breathing attempts as training progressed. Exposed snails to 100 µgL^{-1} Cu were, instead, not able to learn or form memory during the training sessions, thus no LTM was formed at the Mt session (n = 27 for control, n = 9 for 50 µgL^{-1} Cu and n = 7 for 100 µgL^{-1} Cu; error bars are SEM). Asterisks indicate significant differences compared with the control; * $p \leq .005$, ** $p \leq .001$, and *** $p \leq 0.001$

Table 4-1 summarises the results gathered, after exposure to Cu as ionic Cu and CuO NMs, from the respiration behaviour and long-term memory formation endpoints expressed as lowest significant observed effect concentration (LOEC) compared with the control.

Table 4-1 Summarized LOEC of all the endpoints assessed after exposure to ionic Cu or CuO NMs.

	LOEC (30 days)		
	Respiration behaviour		**LTM formation**
	TBT	**TBN**	
$CuSO_4$	40 μgL^{-1} Cu	20 μgL^{-1} Cu	20 μgL^{-1} Cu
Pristine CuO NMs (H_2O)	100 μgL^{-1} Cu	100 μgL^{-1} Cu	100 μgL^{-1} Cu
CuO-PVP(H_2O) NMs	200 μgL^{-1} Cu	200 μgL^{-1} Cu	200 μgL^{-1} Cu
CuO-ASC(H_2O) NMs	100 μgL^{-1} Cu	200 μgL^{-1} Cu	100 μgL^{-1} Cu
CuO(PO_4^{3-}) NMs	100 μgL^{-1} Cu	50 μgL^{-1} Cu	50 μgL^{-1} Cu
CuO-PVP(PO_4^{3-}) NMs	50 μgL^{-1} Cu	50 μgL^{-1} Cu	n/a
CuO-ASC(PO_4^{3-}) NMs	200 μgL^{-1} Cu	200 μgL^{-1} Cu	n.d.

4.4 Discussion

This study investigated the effects of CuO NMs and Cu^{2+} ions on the ability of *L. stagnalis* to form long-term memory (LTM) following operant conditioning of aerial respiration. Furthermore, the influence on toxicity of different coatings and functionalization medium was evaluated together with an assessment of how toxicity differed from the toxicity of the pristine CuO NMs.

In the light of previous results (Chapter 2 and 3), it was hypothesized that Cu ions as $CuSO_4$ would trigger a stronger stress response than the CuO NMs on the snails through blocking the cognitive abilities of the snails and that, overall, cognitive impairment would give an early signal of toxicity to exposure to NMs compared to the more conventional L/EC50.

However, the initial hypothesis was only partially confirmed, since the LTM test indicate toxicity at concentrations lower than the L/EC50 found in Chapter 3 for all the materials investigated except for CuO-ASC NMs functionalised in either PBS or Milli-Q water (Tab. 4-1). Indeed, cognitive abilities of the snails were affected at lower concentrations than the LC50 estimated; however, when examining the other endpoints evaluated (chapter 3, section 3.2.2 and 3.2.3), the alteration of the feeding behaviour

and the fecundity (eggs) of the snails these were found to be a more sensitive endpoint than LTM when exposed to either CuO-ASC(H_2O) NMs or CuO-ASC(PO_4^{3-}) NMs.

Aerial respiratory behaviour in *L. stagnalis* can be operantly conditioned such that the animal learns not to perform the behaviour; however, the training procedure might change depending on the strain of snails tested. Different studies (Inoue et al. 1996, Parvez et al. 2006, Dalesman et al. 2011, Teskey et al. 2012, Lukowiak et al. 2014, Hughes et al. 2017) have demonstrated that different strain of *Lymnaea* exhibits different cognitive abilities such as that to successful operant conditioning the snails, and so different training procedures may be followed. Thus, snails have been defined as: "smart" when they are able to form LTM following a single 0.5 h training session, *e.g*. Chilton Moor strain (Dalesman et al. 2011), or "average" when a single 0.5 h training session only results in a 3 h memory (intermediate-term memory (ITM)) and to form LTM two training session set apart by 1 h are needed, *e.g.* Dutch snails (Lukowiak et al. 1996), such as those tested in this research project (Fig. 4-12, 4-13, 4-14, 4-15, 4-16 and 4-17).

The specific strain of the snail will determine how it responds to a specific stressor, such as pollutants, crowding or predator presence (Lukowiak et al. 2014).

"Average snails" tested in Young et al. (2017), after exposure to three sublethal concentrations of Ag NMs (5 , 10 and 50 μgL^{-1}) and Ag ions (1 , 5 and 10 μgL^{-1}), exhibited a memory formation as an inverted U-shaped curve of the Y-D/H law. At lower concentrations, neither the Ag NMs nor the Ag ions affected LTM formation. However, at intermediate concentrations both substances enhanced the ability of snails to form memory. And finally, at higher concentrations memory formation was blocked.

In contrast, in this research project, exposure to Cu^{2+} ions as $CuSO_4$ at both concentration tested of 20 and 40 μgL^{-1} Cu, resulted in the formation of memory after one day of training, however snails were not able to retain that formed memory for more than 24h due to the additional stress imposed by the poking, which snails appeared to not be able to overcome, when they already exhibited a change in the normal homeostatic behaviour (Fig. 4-4, 4-5 and 4-12).

Learning and memory are two separate and distinct processes each with different underlying molecular mechanisms and forms of expression. Learning can be defined as the acquisition of a new behaviour, while memory is defined as the ability to both store and recall the new information. The consolidation of the memory, which is the process that leads to the formation of memory following learning can be modified or blocked entirely if a stressor is applied (Lukowiak and Dalesman 2013, Lukowiak et al. 2014).

Thus it appeared that $CuSO_4$ interfered with the consolidation of the memory that at molecular level is dependent on both transcription and *de novo* protein synthesis (Parvez et al. 2005), such HSP_S protein (Foster et al. 2015, Sunada et al. 2016) or molluscan insulin-related peptide II and protein kinase C which play a role in the LTM formation of conditioned taste aversion (Azami et al. 2006, Takigami et al. 2014).

Indeed, results showed that the exposure to Cu ions altered significantly the aerial respiration behaviour, after 30 days of exposure, at both exposure concentrations (20 and 40 μgL^{-1}, decreasing the number of successful pneumostome opening when no stimulus was applied (Fig. 4-4 and 4-5). Therefore, further studies are needed to discern if the significant effects observed during the Mt tests might be solely attributed to the detrimental impact of copper on the snails' cognitive ability.

For instance, Byzitter et al. (2012) when exposing *L. stagnalis* to a mixture of heavy metals (Zn, Cd) concluded that the block of memory recorded was only a result of the snail sensing these metals in the pond water and not to a direct physiological effect on the central nervous system (CNS). However, results showed that, in contrast to this research study, snails did not exhibit any significant change in respiration behaviour during exposure, indicating a possible different mechanic of toxicity of Cu compared with the metal used in Byzitter et al. (2012).

O'Gara et al. (2004) demonstrate that copper exposure adversely affected the ability of worms, *Lumbriculus variegatus*, to produce body reversal or helical swimming behaviours following tactile stimulation due to an adverse effect on their neural functions. This was demonstrated by a reduction of the giant fibre conduction velocity, which activate motor neurons, which in turn activate longitudinal body wall muscles that respond to stimulation of the posterior three-fourths of the worm's body (O'Gara et al. 2004).

A similar conductivity reduction could have played a role on the showed reduction of aerial respiration in *Lymnaea*. Indeed, the aerial respiratory neuron central pattern generator in *L. stagnalis* consists of three identified interneurons: Right Pedal Dorsal 1 (RPeD1) which initiates the respiratory rhythm receiving inputs from the pneumostome area to modulate its activity; and the Visceral Dorsal 4 (VD4) and the Input 3 Interneuron (IP3I) which, respectively, initiate the opening and closure of the pneumostome by excitation of close motor neuron (Lukowiak and Syed 1999).

Finding gathered from the exposure to pristine CuO NMs revealed, as expected, a lower toxicity compared to $CuSO_4$. Indeed, snails exposed to the lower concentration 50 μgL^{-1} Cu, showed no alteration in respiration behaviour and on the capability to learn and

form memory. At the concentration 100 μgL^{-1} Cu, snails exhibited, in contrast, a significant decrease on the total time spent performing aerial respiration after 30 days of exposure, together with a decrease in pneumostome opening after 20 days of exposure (Fig. 4-6 and 4-7). Data from the LTM test showed that, although snails significantly reduced the TBN as the training progressed; snails were not able to form LTM, as measured at 150 μgL^{-1} Cu (Fig. 4-13).

In vivo studies have also shown that CuO NMs can accumulate in the brain of rats and have a high capacity to impair cognitive abilities due to neuronal damage of the hippocampus and to induce vacuoles formation, detachment from the substratum, and disruption of the neurite network of the somatosensory neurons in dorsal root ganglia (Prabhu et al. 2010, An et al. 2012).

In fish, Al-Bairuty et al. (2013) comparing the histopathological effects of $CuSO_4$ and Cu NMs to rainbow trout after 10 days of exposure, showed that at same exposure concentrations, Cu NMs provoked similar although more severe brain pathologies. Furthermore, Sovová et al.(2014) demonstrated that Cu NMs had an stronger adverse effect on the olfactory-mediated anti-predator behavioural response of rainbow trout compared to than with the equivalent nominal total concentration of Cu in the form of a metal salt ($CuSO_4$).

In contrast, in this research study, $CuSO_4$ elicited a greater toxicity response than CuO NMs, even when considering the 60 % dissolution of pristine CuO NMs in these experimental conditions (see chapter 3, section 3.3.1). Indeed, when exposing snails at 50 μgL^{-1} Cu of pristine CuO NMs, which corresponded to 30 μgL^{-1} Cu of Cu ions in solution, snails were able to learn and form memory while within that concentration range; snails were not able to form memory if exposed to ionic Cu^{2+}. This indicated, as previously suggested, that in the case of CuO NMs the mechanism of toxicity could not be attributed solely to the dissolved ions but most likely to a specific nanoparticulate effect.

Whilst the possibility that the impairment of the cognitive abilities recorded in this research study, could be due to a further suppression in the respiration behaviour, data gathered from exposure to SbyD NMS suggest that this correlation might not be that simple.

For example, when exposing snails to CuO(PO_4^{3-}) NMs, results obtained were in agreement with previous data (see Chapter 3). An increasing in toxicity of the pristine CuO NMs was recorded due to the presence of the PBS in solution, which translated to a concentration-response observed on the respiration behaviour at the lowest

concentration tested of 50 μgL^{-1} Cu (Fig. 4-8 and 4-9). At this concentration, snails were still able to form memory and thus to change aerial respiration behaviour after 1 day of training; however, the memory was not retained, resulting in failing to form memory at Mt test session (Fig. 4-14).

A similar response it was found in the experiment carried out using CuO-PVP(H_2O) NMs. When exposed to the highest concentration of 200 μgL^{-1} Cu, snails were still able to form memory at the second day of training (Fig. 4-15), and due to the decrease in their respiration activity after 20 days of exposure (Fig. 4-10); snails were not able to retain the formed memory for more than 24h. At the lowest and intermediate concentrations (50 and 100 μgL^{-1} Cu), the snails' respiration behaviour was normal during the 30 days of exposure and snails were able to form long term memory which lasted more than 24 h. In contrast, LTM tests using CuO-PVP(PO_4^{3-}) NMs could not be performed due to the extremely impairment of the respiration activity of the snails for all the treatment during the 30 days of exposure.

For example, Podolski et al. (2005) demonstrated that the complex fullerene C_{60}/PVP, injected into the hippocampus of rats, prevented the disturbance of long-term memory consolidation induced by cycloheximide, a protein synthesis inhibitor which interferes with the translocation step in protein synthesis blocking translational elongation. Authors used C_{60} due to its known neuroprotective function; they found that when complexed with PVP, C_{60} increases its efficiency on adsorbing the inhibitor cycloheximide (Zaporotskova and Chernozatonskii 2005), allowing so the rats to learn and form long-term memory. However, this result was observed only when a 10000-molecular weight PVP was used. With the increase of PVP molecular mass, the efficiency of C_{60}/PVP significantly decreased. At PVP molecular mass of 25,000, the complex did not protect the LTM consolidation against cycloheximide.

In this research project, to coat the pristine CuO NMs, PVP with molecular weight of 28000 was used. It is so possible, that in this instance, the presence of PVP mitigated the neurotoxicity effect of the core pristine CuO NMs, which was displayed at 100 μgL^{-1} Cu (Fig. 4-13 and 4-14). Thermal analysis confirmed that due to the presence of PBS in CuO-PVP(PO_4^{3-}) NMs, the quantity of PVP attached to the core CuO NMs was lower than in CuO-PVP(H_2O) NMs. Therefore, it is likely that this might be the main factor contributing to the remarkable difference in toxicity between the two NMs. To corroborate this hypothesis, it would be interesting to test lower concentrations of CuO-PVP(PO_4^{3-}) NMs, which do not cause mortality, to verify the potential protection action of PVP against the neurotoxicity of the pristine CuO NMs.

Finally, results gathered from the exposure to the either CuO-ASC NMs showed similar effect on the ability of the snails to learn and form memory. Indeed, when snails were exposed to 200 μgL^{-1} Cu of either CuO-ASC(PO_4^{3-}) NMs or CuO-PVP(H_2O) NMs, it was not possible to test snails for LTM due to their strong decrease in respiration activity. However, contrary to pristine CuO NMs at 100 μgL^{-1} Cu, snails were still able to retain the formed memory for more than 24h when exposed to CuO-ASC(PO_4^{3-}) NMs and to form memory after one day of training in the presence of both NMs (Fig. 4-16 and 4-17). At 50 μgL^{-1} Cu, the snails' cognitive abilities and respiration behaviour were not affected.

It is suggested that the presence of ascorbate in solution, a known antioxidant, mitigated the toxicity effect of the core pristine CuO NMs reducing the formation of ROS; which as other authors demonstrate (An et al. 2012, Prabhu et al. 2010, Bulcke et al. 2017), are inducers of neurotoxicity in organisms. Indeed, several studies have demonstrated that when mice were exposed to toxicant, if pre- or co-treated with ascorbate, ameliorative effects were detected on the cognitive abilities (Socci et al. 1995, Ambali et al. 2010, Stansley and Yamamoto 2015).

Nevertheless, overall, we cannot still reject the possibility that the cognitive toxicity effects showed during the memory test formation were due not solely to the demonstrated copper toxicity (see Chapter 2 and 3) but also to exposure to repetitive hypoxic environment in such short distance period (1 hour). Indeed, previous studies have demonstrated that the ability of *Lymnaea* to perform aerial respiration is not protective safeguard from detrimental chronic hypoxia effects (Hoefnagel and Verberk 2017).

For example, Fei and Feng (2008) exposing snails to chronic hypoxia found neurobehavioral dysfunction already after 2 days of exposure. Snails exposed to the hypoxia environment ($\approx$ 5% O_2) revealed an alteration of the righting reflex (resume upright posture) and a slowed light response (extension of the tentacles) compared to those exposed to a normoxic aquatic environment. Authors attributed these changes to a deficiency in either muscle and/or neuronal functions as demonstrated by the downregulation of synaptic proteins involved in the calcium-dependent exocytosis (*e.g.* synaptotagmin and syntaxin) of neurotransmitters (Fei and Feng 2008). In accord, Hoefnagel and Verberk (2017) showed detrimental effects due to long-term hypoxia exposure. Snails attempted to compensate for chronic poor water oxygenation by increasing respiratory surfaces of their tentacles, which are an important site for gas exchange, to minimise time spent surfacing and increasing ventilatory behaviour

(opening and closing of pneumostome) (Taylor et al. 2003). Previous studies have demonstrate that exposure to copper determine an alteration of the normal function of sensory and respiratory organs (Byzitter et al. 2012, Al-Bairuty et al. 2013, Koopman et al. 2016). It is so possible that the data gathered from the memory formation tests were a result of a synergetic effect of the exposure to Cu (ionic or nano) and hypoxic environment.

4.5 Conclusions

To conclude, this study documents the adverse effects of copper exposure, either as salt or in the nano form, on the cognitive abilities and respiration behaviour of the pond freshwater snail, *Lymnaea stagnalis*, setting the experimental results and approach towards the use of these endpoints as non-invasive methods to assess the toxicity of NMs.

Indeed, overall both endpoints used gave an early toxicity indication of Cu compared with the more conventional LC50 approach. Furthermore, tests with SbyD CuO NMs indicated that coating pristine CuO NMs with PVP in water, would greatly ameliorate the capability of snails to learn and form memory.

The adverse effects of Cu (ionic and nano) exposure on the neural function noted in this study would be expected to reduce the ability of *Lymnaea* to escape from predators and thus resulting in an impact on the population dynamics of this species.

However, further studies are needed to discern the change in behaviour from the possible physiological processes occurred in response to Cu exposure.

Chapter 5 Modulation of antioxidant and detoxification gene expression in juveniles of *L. stagnalis* exposed to CuO NMs

5.1 Introduction

Different studies have indicated the higher toxicity of NMs and heavy metals to the early stages of development of organisms compared to their adult stage (Grosell et al. 2006, Grosell and Brix 2009, Brix et al. 2011, Brix et al. 2012, Munley et al. 2013, Niyogi et al. 2014). Higher toxicity has been manly attributed to the higher feeding rate of the juveniles concurrent with faster ions uptake rate to promote the organism's development (Croteau et al. 2014a, Croteau et al. 2014b). Indeed, in many species, in short-term exposures, metal uptake by smaller individuals is faster (μg g^{-1}) than uptake by large individuals, resulting in a negative correlation in metal concentration and body size (weight) (Strong and Luoma 1981).

For instance, Madhav et al. (2017) in a comparative study of the acute lethal toxicity of CuO NMs to different life stage (1, 2, 7 days old and adults) of the crustacean, *Artemia salina*, found that its early life stages were greatly affected compared with the adults. In particular, authors showed that the 7d old stage was, overall, the most sensitive due to their higher voracity compared to the adult crustacean and to the poor development of digestion and excretory systems in nauplii (1 and 2 days old).

Furthermore, Bicho et al. (2017) showed in a full life-cycle experiment that effects of CuO NMs and $CuCl_2$ on the enchytraeid, *E. crypticus*, were life stage dependent. Indeed, CuO NMs caused toxicity during the juvenile stage, showing reduced growth, maturation and hence reproductive output in the adults. In contrast, $CuCl_2$ induced a higher toxicity during embryo development and/or affected hatching success which is reflected in their survival when adults and consequent decreased reproduction.

Similar dichotomy between juveniles and adults was found, when assessing the toxicity of different chemicals on the pond snail, *L. stagnalis*. For instance, Croteau et al. (2014a) evaluating the toxicity and uptake of Ag NMs at environmentally relevant exposures, highlighted the influence of the snail body size on uptake rate constants. The rate constant of Ag uptake from water was faster for smaller than for larger size snails, for either Ag ions or Ag NMs coated with citrate. Furthermore, Brix et al (2011) attributed the higher sensitivity in terms of growth inhibition, of the juveniles of *L. stagnalis* to Cu ions compared with adult stages, due to the disruption of Ca homeostasis caused by Cu. Indeed, *L. stagnalis* in its early life stages requires high Ca intake to sustain the rapid shell formation and so very large growth rates corresponding to around 20% of body mass per days (Crémazy et al. 2018).

This information, combined with the results gathered by the previous experiments (see chapter 2 and 3), was used to justify the next part of the study in which a sub-chronic toxicity experiment using juveniles of *L. stagnalis* was performed. Based on the hypothesis that toxicity of CuO NMs was mainly due to a nanoparticulate effect, that is, divergent mechanisms of toxicity from the ionic Cu may occur, transcriptional responses of genes responsive to environmental stress or metal exposure were investigated in juveniles of *L. stagnalis*.

Copper and CuO NMs are known to generate reactive oxygen species (ROS) (*e.g.* superoxide ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$) and singlet oxygen (O_2)) via Haber–Weiss and Fenton reactions and thus, to provoke intracellular oxidative stress when the organism is not able to detoxify or to repair the resulting damage (Gomes et al. 2012). The cellular damage can consist, for example, in denaturation of proteins, mutation of DNA and lipid peroxidation (Siddiqui et al. 2015, Ivask et al. 2014). However, the extent of the contribution of the CuO NMs and the dissolved Cu ions to the production of reactive oxygen species (ROS) is still controversial, inasmuch as it is influenced by the different size, shape and physico-chemical properties of the chemical, eliciting a diversity of results between organisms and NMs (Soni et al. 2015).

Production of moderate levels of ROS plays specific roles in the modulation of several cellular events, including signal transduction, proliferative response, gene expression, and protein redox regulation. High ROS cellular levels, in contrast, which can be caused, for example, either by uptake of formed ROS in the exposure medium or by interaction with internalized NMs (Torres-Duarte et al. 2016), are indicative of oxidative stress damage.

As defence mechanisms, organisms can produce enzymes, such as catalase (CAT) and superoxide dismutase (SOD), which can convert harmful ROS to a less harmful compound (Siddiqui et al. 2015). SOD is the first defence response to ROS catalysing the dismutation of superoxide anion radical, $^{\bullet}O_2^-$, to water and H_2O_2, which CAT then reduces to H_2O and O_2 for elimination of ROS (Atli and Grosell 2016), preventing oxidative stress and maintain cell homeostasis (Ali et al. 2012).

Along with antioxidant enzyme protection, stress proteins (also called heat-shock proteins (HSPs)) operate as molecular chaperones to protect and repair stress-damaged proteins (refolding). These proteins can be induced to protect the organisms from stress (*e. g.* oxidative stress, thermal stress and anoxia) and harmful conditions (*e.g.* heavy metals and xenobiotics) (den Besten and Munawar 2005, Foster et al. 2015). In

particular, HSP_{40} proteins represent a large family whose major function is to regulate adenosine triphosphate (ATP)–dependent polypeptide binding by another HSP protein, the HSP_{70}. The interaction of a single HSP_{70} with multiple HSP_{40}s generates HSP_{70}-HSP_{40} pairs that prevent protein misfolding and aggregation at distinct locations within the cell (Fan et al. 2003).

A special group of stress proteins is the metallothioneins (MTs), responsible for the implementation of detoxification mechanisms in conditions where significant amounts of heavy metals exist (den Besten and Munawar 2005). There are two MT isoforms, MT1 which participates more in physiological activities, whereas MT2 binds nonessential metals, such as cadmium and copper (Homa et al. 2016).

CuO NMs and ionic Cu induce the expression of these stress related and environmental exposure proteins, as evidenced by enzymatic activity or transcriptional modulation. Such effects have been observed in almost all levels of biological organisation, spanning plants, bacteria and mammalian cell lines in vitro (Ivask et al. 2014).

For instance, Griffitt et al. (2007) examined the acute toxicity of CuO NMs (1500 μgL^{-1} Cu) or soluble Cu on the gill of zebrafish, to distinguish their role on the acute toxicity recorded ($LC50_{48h}$ 1500 μgL^{-1}). Results indicated that, overall, stress and metal responsive genes in the gill were transcribed to higher levels in fish exposed to CuO NMs compared to ionic Cu, although the same concentration of Cu ions was present in solution. This indicates that the effects of CuO NMs were not mediated solely by dissolution. In particular, fish exposed to CuO NMs showed a higher fold induction for hypoxia-inducible factor 1 (HIF-1), HSP_{70} and copper transport regulatory protein (CTR) compared to those exposed to ionic copper alone (Griffitt et al. 2007)

Similarly, Siddiqui et al. (2015) found an higher induction of antioxidant system after exposure to CuO NMs compared to ionic Cu as $CuCl_2$. Authors investigated and compared, however, the effects of 21 days exposure to CuO NMs and $CuCl_2$ at much lower concentrations of 10, 50, and 100 μgL^{-1} Cu on the sea anemone, *Exaiptasia pallida*, quantifying the activities of antioxidant enzymes such as CAT, glutathione peroxidase (GPx), glutathione reductase (GR) and carbonic anhydrase. As a consequence of increased copper exposure, in either form, anemones increased activities of all these antioxidants. Exposure to 50 and 100 μgL^{-1} Cu of CuO NMs or ionic Cu, elicited an increase over time on the CAT activity. In anemones exposed to 10 μgL^{-1} Cu of CuO NMs, in contrast, CAT increased over time with significant elevations observed at 7 and 14 days; and then decreased back to control levels by 21 days. These results

suggest that the oxidative stress response is dependent on the form of copper, as well as, concentration and perhaps exposure time (Siddiqui et al. 2015).

Similarly high sensitivity of CAT and glutathione S-transferase (GST), as biomarkers for oxidative stress due to Cu exposure as CuO NMs or Cu^{2+} (10 μgL^{-1} Cu), was found by Mouneyrac et al. (2014). Authors, reviewed the results of two sub-chronic (16 days) experiments (seawater and microcosm) conducted by Buffet et al. (2011, 2013), using the bivalve mollusc *S. plana,* and the annelid polychaete *H. diversicolor*. Results showed that in the mollusc, GST and CAT enzymes were the most responsive to exposure, compared to SOD and MT, in microcosms, and they were more sensitive to CuO NMs compared with the soluble form of Cu. Nevertheless, in both experiments, a significantly high induction of MT, due to either forms of Cu, was detected. Finally, when organisms were exposed via spiked seawater, SOD activity was significantly higher in bivalves exposed to ionic Cu compared with CuO NMs. In contrast, SOD activity showed no responses in *H. diversicolor* after exposure to both forms of metals. For both experiments, no cellular damage due to oxidative stress was observed, indicating that the antioxidant system was efficient in the removal of ROS, precluding further damage of the cells (Mouneyrac et al. 2014). In contrast, Gomes et al. (2011) exposing the mussel, *M. galloprovincialis*, to the same concentration of CuO NMs and ionic Cu (10 μgL^{-1} Cu), found that both chemicals originated lipid peroxidation despite the highly induced oxidative defence observed in gills, for example, after the exposure to CuO NMs within the first week of exposure. Moreover, like the aforementioned studies, an induction of MT was detected throughout the exposure in mussels exposed to nano and ionic Cu, with a slighter increase when exposed to CuO NMs (Gomes et al. 2011).

Therefore, in this research study, it was hypothesized that after sub-chronic exposure, high transcriptional induction of genes related to stress and metal exposure (HSP_{40}, MT, CAT and SOD) would occur as a protective response to ROS generated both by exposure to copper ions in solution and those released from the CuO NMs (pristine CuO NMs, SbyD CuO NMs (PVP and ASC) and CuO Fragmented Products) in juveniles of *L. stagnalis.*

Furthermore, snails were subjected to additional stress represented by a thermal shock, to evaluate the response of the stress system when already subjected to chronic exposure to toxicants. Indeed, in a recent study Salo et al. (2017) analysed the single and combined effects of experimental heat waves (8 days of 23.5°C) and micropollutants (identified as a mixture of low concentrations of pharmaceuticals, oestrogenic hormone,

biocides, corrosion inhibitor, and artificial sweetener) on *L. stagnalis* on a broad range of traits, such as, for example, respiration rate, feeding performance and immune responses. Findings showed heat wave-induced metabolic imbalances which lead to trade-offs between competing functions such as reproduction and immune defence, whereas constant pollution load primarily reduced fecundity. However, a quick recovery was recorded after 8 days from the heatwave. Furthermore, the interaction of heat-wave and pollutants resulted in strong combined effects on snails' fecundity, while the single effects of the studied stressors were less pronounced on any of the studied traits and only affected food consumption, respiration and growth marginally (Salo et al. 2017).

As reviewed in previous chapters, in freshwater snails, ionic Cu and CuO NMs have shown to cause toxicity at different biological levels; however, few of these studies assessed their effects on oxidative stress and none assesses the induction of gene expression of stress related genes due to exposure to these chemicals.

Atli and Grosell in 2016 published one of the most comprehensive and recent studies evaluating the response of antioxidant systems in the tissues of *L. stagnalis* during acute copper exposure. These authors measured the response of enzymatic (SOD, CAT, glutathione peroxidase (GPx) and glutathione peroxidase reductase (GR) and non-enzymatic responses (glutathione, GSH, oxidized glutathione, GSSG and GSH/GSSG ratio) following acute ionic Cu toxicity exposure, as $CuSO_4$ (2 – 90 μgL^{-1} Cu for 96 hrs) in the hepatopancreas, foot muscle and mantle tissues of *L. stagnalis.* Results showed an antioxidant response to acute Cu exposure at concentration as low as 2 μgL^{-1} Cu. In particular, GPx and CAT were the most responsive antioxidant enzymes to Cu exposure with activity increasing with copper concentration. In addition, authors identified that in the hepatopancreas, followed by the foot muscle, antioxidant enzymes exhibited the greatest changes, with the mantle showing only subtle responses at the highest concentrations tested of 90 μgL^{-1} Cu. Total and reduced GSH increased in hepatopancreas but decreased with GSH/GSSG ratios at all Cu concentrations in the foot muscle (Atli and Grosell 2016).

The only other study available, evaluating the modulation of the antioxidant enzymes in the genus *Lymnaea* after exposure to CuO NMs was by Ali and Ali (2015). These authors exposed adults of *L. luteola* for 5 days to sublethal concentrations of CuO NMs (7 and 12 μgL^{-1} of NMs), and determined the antioxidant response in the hepatopancreas. Results showed, in contrast with the studies described previously, a significant decrease in SOD and CAT activity on the first and the second day respectively of exposure. In addition, GSH, GPx, and GST levels decreased in a

concentration- and time-dependent manner, leading to an increase in both lipid peroxidation and genotoxicity as the time and the concentration of exposure increased. The authors concluded that ROS production was so high that the antioxidant system was not able to impede cellular damage after the first 2 days of exposure (Ali and Ali 2015).

Further evidence about the development of Cu-detoxification mechanisms in ionic Cu exposed *L. stagnalis* after chronic exposure can be found in Ng et al. (2011). Lipid peroxidation and metallothionein-like proteins were measured, after 28 days of exposure, to a concentration range of 0-35 μgL^{-1} Cu. Cu detoxification was shown in this chronic exposure by increases in metallothionein-like (MTL) protein concentrations and Cu binding to metal-rich granules at the highest concentration tested. This was complemented by a decrease in thiobarbituric acid-reactive substances, and changes in the subcellular distribution in the soft tissues, which indicate no oxidative stress damage likely associated with the detoxification action of MTL (Ng et al. 2011).

Finally, no studies have been published so far elucidating the mechanism of induction of HSPs on *L. stagnalis* due to exposure to Cu in either form, although some authors have explored the modulation of these proteins after exposing the snails to a thermal stress (Foster et al. 2015, Sunada et al. 2016). Indeed, *Lymnaea* species living in slow flowing or stagnant freshwater habitats, experience fluctuating temperatures within a year, varying from 0 ˚C (in winter some molluscs may even survive in ice) to 35 ˚C (on hot summer days), however the optimum living temperature is around the 20 ˚C (Sidorov 2005).

For instance, Foster et al. (2015) profiled the time-related expression for HSP_{40} and HSP_{70} proteins on the CNS of adults of *L. stagnalis* after acute heat shock exposure (1h, 30 ˚C). Results indicated that exposure to an acute thermal stress was sufficient to increase the synthesis of HSPs above constitutive levels in *L. stagnalis*. Indeed, both transcripts were rapidly induced within the first 30 min after heat shock, with a higher induction of HSP_{70} (<100-fold relative to control) compared with HSP_{40} (<20-fold relative to control). Expression levels of HSP_{40} returned to control levels within 8 h after the heath shock, in contrast to HSP_{70} which levels decreased significantly but stayed well above control level (<20 fold) after 8h of recovery. Thus, it appeared that snails up-regulated HSPs in order to increase their thermos tolerance (Sunada et al. 2016) and thus limiting the stress damage in the cells caused by the suddenly increased temperature.

5.2 Materials and Methods

5.2.1 Test chemicals and nanomaterial characterisation

A $CuSO_4*5H_2O$ stock solution was prepared, immediately before use by dissolving the appropriate amount of metal powder in Milli-Q water with a grade of 18.2 MΩ cm.

Pristine CuO NMs (nominal size 20nm) stock suspension was prepared by dispersing the required amount of CuO NM powder in 100 ml of Milli-Q water and then bath sonicated for 8 minutes twice, with ~10 seconds manual shaking in between, following the protocol of Jacobsen et al. (2010).

From the SbyD CuO NMs available, only those suspended in Milli-Q water were selected due to their higher physico-chemical stability in OECD 203 medium compared with those suspended in PBS (for further details, see Chapter 1, section 1.2.3). Stock suspensions of SbyD CuO NMs coated with coating agents PVP and ASC, provided by ISTEC-CNR, Faenza (IT), were synthesised in Milli-Q water at the concentration of 10 gL^{-1} Cu. To prepare working stock suspensions, the provided stocks were vortexed thoroughly before dilution (1:100) in the culturing medium, OECD 203 (OECD 1992).

Nanomaterials characterisation was performed by third parties, such as the University of Venice and ISTEC-CNR, which consisted of (see Chapter 1, section 1.2.3 and Chapter 2, section 2.2.1 for further details):

- dynamic light scattering to measure hydrodynamic diameter (d_{DLS});
- electrophoretic light scattering (ELS) to determine zeta potential (ζ-pot_{ELS});
- transmission electron microscopy (TEM) to obtained images and size measurements for pristine CuO NMs;
- centrifugal separation analysis (CSA)to analyse velocity of sedimentation of the SbyD NMs;
- ICP-OES to calculate the dissolution of Cu ions from the three different CuO NMs after dilution in Milli-Q water and OECD medium.

CuO_Acryl_FP were produced by third party within the SUN (BASF and EMPA) by incorporation of the pristine CuO NMs in products of the SUN industrial partners and subsequently mechanically disrupted to generate Fragmented Products (FP), to mimic the use-phase (see Chapter 1, subsection 1.2.3.3 for more details about the fragmentation method). Due to the restriction of the amount of FP material produced, only characterisations of size and ions release (in Gamble's medium) were performed.

5.2.2 Experimental design

Pilot studies, following the protocol applied in the acute lethal studies described in Chapter 2, were performed to verify if the acute lethal toxicity of CuO NMs would change after an acute exposure (96h) at higher temperature (29 °C).

Furthermore, acute lethal exposure to CuO_Acryl_FP using juveniles of *L. stagnalis* was also performed, to estimate $LC50_{96h}$ necessary to determine the sublethal concentrations for the long-term exposure.

A long-term exposure design using juveniles (7-9 days old, ≈ 1-2 mg ww) snails was developed following the protocol described in Niyogi et al. (2014) and Foster et al. (2015). The experiment consisted in 24 replicates per sampling (after heat shock and after 4h recovery from the heat shock), 10 snails per replicate. Experimental design included a negative control (medium only); 2 positive controls (only Cu, no heat shock) and only medium and heat shock) and a treatment (Cu and heat shock) (Fig. 5-1). Furthermore, 3 replicates were sampled prior to the start of the experiment (0h) for each exposure time point selected (24h, 96h and 10d).

Snails were placed in 250 ml polypropylene containers with 240 ml of test medium under static-renewal conditions at 20 ºC. Container lids were perforated before starting the experiment to facilitate oxygen exchange. Snails were fed lettuce daily *ad libitum*, every 2 days and 100% medium change was performed, and mortality recorded.

Snails were exposed to sublethal concentrations of the different CuO NMs, with the sublethal concentration calculated based on ¼ and 1/8 of the LC50 values obtained from the acute lethal experiments previously performed (see Chapter 2). For $CuSO_4$ for which the LC50 determined was very low (5.6 μgL^{-1} Cu), the sublethal concentrations used were ½ and ¼ of the LC50 value.

After specific time points (24 hrs, 96 hrs and 10 days) selected replicates were subjected to a thermally stressful condition (30 °C for 1 h) and sampled just after the heat shock after 4 hours of recovery from the heat shock. Modulation of 4 genes responsive to environmental stress or metal exposure were then assessed (SOD, CAT, MT and HSP_{40}) (Fig. 5-1), in order investigate if Cu in ionic or nano form induced similar or divergent transcriptional responses in *L. stagnalis*.

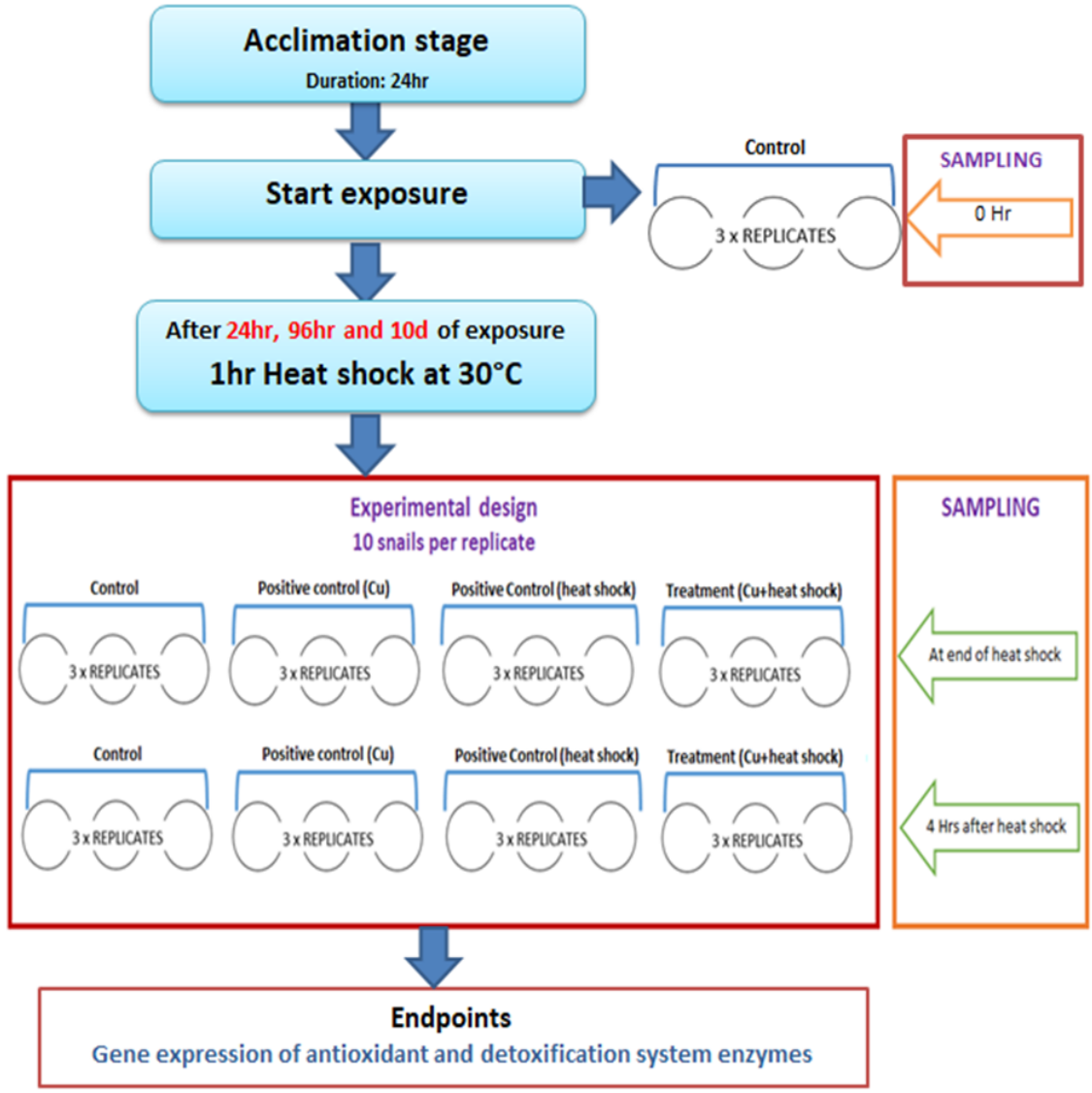

Figure 5-1 Experimental design of chronic exposure of juveniles of *L. stagnalis* under thermal stress condition.

5.2.3 Total RNA extraction and gene expression analysis

Snails' replicates (pool of 10 snails per sample) were immediately frozen at -80 °C after sampling. RNA was extracted from a pooled sample of 10 snails (whole body) using the TRI Reagent® (Sigma-Aldrich©) method following the manufacturer's protocol. Traces of genomic DNA were removed using a DNase treatment performed following the instructions given within the DNase kit (Primerdesign Ltd) used. Quality of total RNA was determined using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, LLC). Only samples that met the quality criteria (260/280 ratio >2.0) were used for further analyses.

RNA samples were stored at -80 ºC until required. Reverse transcription (RT) reaction was performed by diluting the sample to 100 ng mL^{-1} and 400 ng of RNA was used to synthesize cDNA following the manufacturer's protocol for the nanoScript 2 Reverse Transcription kits (Primerdesign Ltd). cDNA was prepared under the following

conditions: annealing at 65 °C for 5 minutes, extending at 55 °C for 20 minutes, and heat-inactivating transcriptase at 75 °C for 15 minutes. cDNA was stored at -80 °C until PCR or q-RT-PCR gene expression analyses were performed. Antioxidant and detoxification enzymes primers were selected using Primer Blast (NCBI) (Tab. 5-1).

Table 5-1 *L. stagnalis* gene specific primers for HSP_{40}, SOD, CAT, MT and a housekeeping gene (beta tubulin, ß-tub). Reference numbers from NCBI, product length in base pairs (bp).

Gene	Ref. Num.	Sequence	Product (bp)	Ann. Temp. (°C)
HSP_{40}	DQ278442.1	*Forward (5'-3'):* ATGTTAAACCTGGATGGAAGGAGG *Reverse (5'-3'):* GCAGGCACGTTTTGCGGTGTTT	79	62
MT	KT253648.1	*Forward (5'-3'):* CTGTAAGTGTGGTGAGGGCTCCAG *Reverse (5'-3'):* TTCCTTTGCTACCTGC	96	60
SOD1	AY332385.2	*Forward (5'-3'):* CGGGAACAATCACATTTACTCATC *Reverse (5'-3'):* CAGCACTTACACATCCATT	140	60
CAT	FJ418795.1	*Forward (5'-3'):* GCAACAACACCCCAATTTTCTCTG *Reverse (5'-3'):* GACGCAGAGTGAAGAA	133	59
ß -tub	X15542.1	*Forward (5'-3'):* TGGACGAGATGGAGTTCACAGAAA *Reverse (5'-3'):* ATGGATGACTTCTGTTGG	141	60

The amplicons were designed to be checked to avoid secondary structure, self-annealing sites, complementarity, and potential hairpins using Beacon Designer (Premier Biosoft©) and UNAFold (Integrated DNA Technologies, Inc. ©.). Amplicon size was verified on a 2% agarose gel after PCR amplification. For all the optimized primers, qRT-PCR reaction efficiency was determined by comparing the change in CT value for the gene transcript relative to the concentration of the standard, using the following dilutions, 1, 1/10, 1/100, and 1/1000. The efficiency of the PCR reaction was computed from the following equation: $Efficiency = 10^{(-1/slope)}-1$, from the standard curve for each plate. Only efficiencies between 0.85 and 1.2 were used for further analysis (Pfaffl 2001).

To conduct quantitative reverse transcriptase PCR (qRT-PCR), lyophilized primers (Eurofins MWG Operon) were reconstituted to 100 mmol with RNase-free water and mixed with PrecisionPLUSTM Mastermix (Primerdesign Ltd) to give a final reaction concentration of 375 nmol in a 20 µL total volume. Fluorescence was detected (StepOne Real-Time PCR System, Applied Biosystems) over 40 cycles with cycling conditions of enzyme activation: HotStart 2min 95ºC, denaturation 5s 95ºC and annealing 5s 55ºC. For analysis, the cycle threshold was set to 25,000 for all qPCR runs. A standard curve of cDNA template (from a known sample) was run on each plate for each gene to allow for within experiment plate normalization. Comparative quantification, using the efficiency corrected method (C_T method), was used to

determine fold-changes in the genes of interest normalized to the housekeeping gene ß-tub (Park et al. 2006). The housekeeping gene, β-tubulin, was no affected by treatments thus, normalized C_T values (ΔC_T) were obtained by subtracting, in the same sample, C_T value of target genes from that of β-tub. Differences between average ΔC_T of control group and ΔC_T of each sample in all tested groups were expressed as $\Delta\Delta C_T$. The fold differences ($2^{\wedge}\Delta\Delta C_T$) of target gene expression in exposed samples compared to the average of target gene expression in unexposed group (0h control) were calculated.

5.2.4 Data analyses

SigmaPlot© version 13.0 (Systat Software, Inc.) was used to perform all statistical analyses. All data were checked for normality of distribution performing a Shapiro–Wilk's test and for variance homogeneity using the Levene's homogeneity of variance test. Logarithmic transformation was applied in cases where assumptions regarding normality and homogeneity of variance were violated; data shown are mean ± standard error.

Genes were considered differentially expressed when they met both fold-change (≥1.5 fold or ≤0.5-fold) and statistical significance ($p \leq 0.025$) criteria. A two-way ANOVA was conducted to determine the interaction effects of increasing time of exposure and concentrations of Cu, in either ionic or nano form, on the gene expression of the selected genes.

When interaction between the two independent variables was significant ($p < 0.05$), an analysis of simple main effects (effect of one factor level on the different levels of the second factor) for each factor was performed using SPSS, with statistical significance receiving a Bonferroni adjustment and being accepted at the $p < 0.025$ level.

If no significant interaction between the increasing time of exposure and concentration of Cu was revealed, an analysis of the main effect for each factor (without taking in consideration the levels of the second factor) was performed with statistical significance receiving a Bonferroni adjustment and being accepted at the $p < 0.025$ level.

All pairwise comparisons using a Tukey's test were run for each simple main/main effect to assess differences between treatment means, with reported *p*-values Bonferroni-adjusted within each simple main/main effect.

5.3 Results

5.3.1 Characterisation of CuO NMs

Results obtained from secondary characterisation of the different CuO NMs have already been described in detail in Chapter 1, section 1.2.3 and further summarized in Table 3-1 in Chapter 3, section 3.3.1.

Results from the second characterisation of the CuO_Acryl_FP showed a median particle size distribution of 73 µm.

Furthermore, total copper and Cu ions release studies were performed only when dispersed in Gamble's medium (simulated lung fluid) by ISTEC-CNR. This medium has a pH 7.4 similar to the OECD 203 used in these experiments, and on balance, considering also the composition of both media, it was considered that findings are comparable with this research study (Marques et al. 2011). Results showed a very low concentration of Cu ions released from the Acryl_FP sample, representative of the matrix without CuO NMs and negligible presence of Cu ions released from CuO NMs.

5.3.2 Pilot experiments and mortality in long-term studies

Results from the pilot studies revealed no change in acute toxicity of pristine CuO NMs when exposed at 29 °C compared to at the ideal temperature of 20 °C. Indeed, the $LC50_{96h}$ value estimated was 1880 (± 132.23 SE) μgL^{-1} Cu for snails exposed to pristine CuO NMs compared with the $LC50_{96h}$ value of 2198 (± 143.8 SE) μgL^{-1} Cu calculated after exposure to the same NM at 20 °C.

Furthermore, no mortality (< 5%) was recorded after 96h exposure at 20 °C to CuO_Acryl_FP at the concentration range from 0-14000 μgL^{-1}. The highest concentration tested (14000 μgL^{-1}) of CuO_Acryl_FP corresponded to 550 μgL^{-1} of Cu; nevertheless, since the limited amount of material available (see Chapter 1, section 1.2.4.3), it was not possible to test higher concentrations which would induce mortality to 50% of the population. Therefore, sublethal exposure concentrations for the long-term exposure on juveniles of *L. stagnalis* were chosen based on the $LC50_{96h}$ value estimated for the pristine CuO NMs.

After long-term (10 days) exposure no mortality was observed (<5%) in juvenile snails of *L. stagnalis* exposed to $CuSO_4$ and to the different CuO NMs, except for SbyD CuO-ASC NMs. Indeed, after 7 days of exposure to SbyD CuO-ASC, 15% and 26% mortality were recorded for snails exposed to 30 and 60 μgL^{-1} Cu respectively, which increased to 17% and 29% respectively after 10 days of exposure. Consequently, for

this NM pooled snail samples for RNA analyses consisted of 5 snails per sample, in order to ensure that there were at least three replicates to analyse per treatment.

5.3.3 Changes in gene expression in response to sub-chronic exposure studies of CuO NMs

An initial comparative study was performed between pristine CuO NMs and $CuSO_4$ to discern the contribution of dissolved Cu in the activation of the selected genes. Gene expression was evaluated using RT-qPCR.

Contrary to the initial hypothesis of high expression of antioxidant genes due to exposure to $CuSO_4$, results showed no significant activation of the SOD, CAT and MT genes, but increased expression levels of HSP_{40} gene by up to 5-fold at 1.25 μgL^{-1} Cu after 96h of exposure (Fig. 5-2).

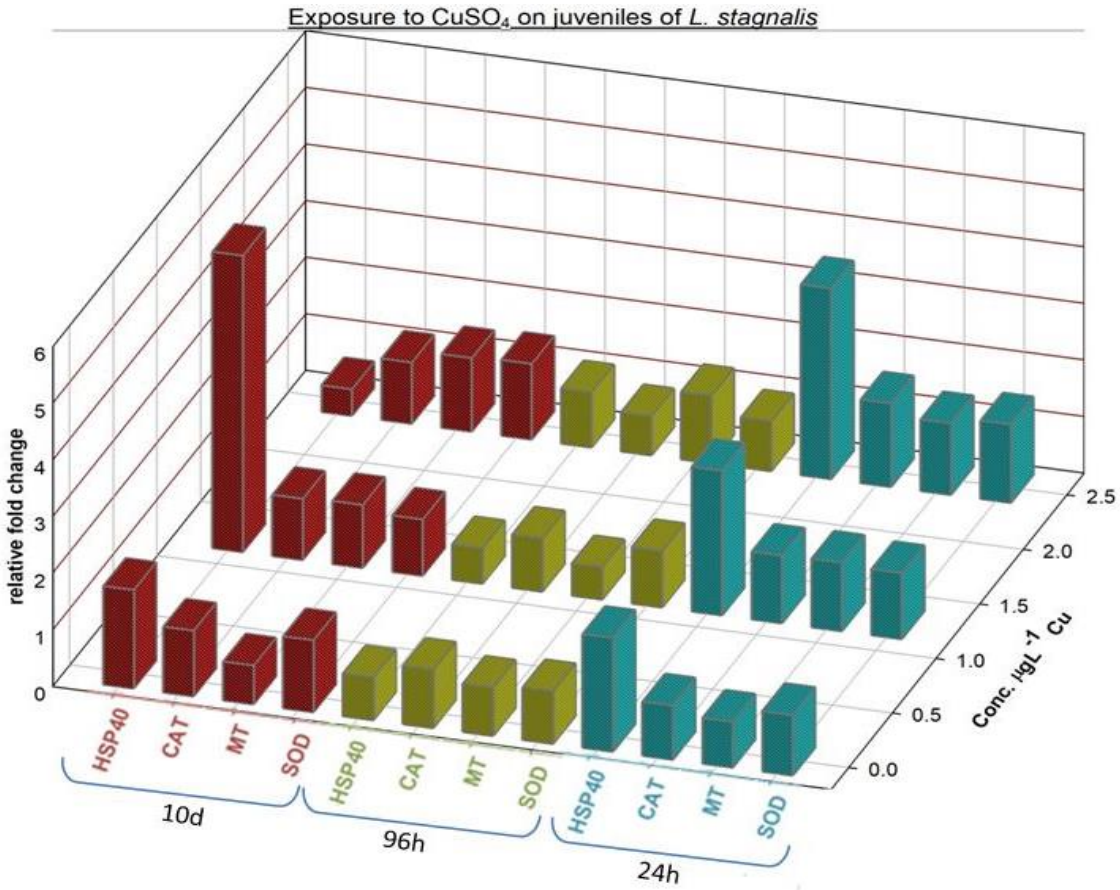

Figure 5-2 Overview of the gene expression levels, relative to 0h time of exposure, of the 4 selected genes along the 10 days of exposure to increasing concentrations of $CuSO_4$ of *L. stagnalis* juveniles. Data are means of 3 replicates per treatment.

A two-way ANOVA was conducted to determine the effects of time of exposure, and concentration of $CuSO_4$ on the gene expression of HSP_{40}. A log10 transformation of the data was computed to meet the condition of homogeneity of variances, which was assessed by Levene's test (p = 0.300). A statistically significant two-way interaction was

detected between increasing time of exposure and concentration of $CuSO_4$, $F_{(4, 37)}$ = 11.9, $p < 0.001$, partial $\eta^2 = 0.56$. Thus, simple main effects for the two factors were assessed and statistical significance was accepted at the $p < 0.025$ level. Simple main effects assessment revealed a statistically significant difference in the expression of HSP_{40} between the concentrations tested after 10 days of exposure, $F_{(2, 37)} = 29.79$, $p < 0.001$, partial $\eta^2 = 0.62$. Furthermore, a significant simple main effect in the expression of HSP_{40} was revealed between the different time point of exposure at the two concentration tested of $CuSO_4$ but not at the control (1.25 μgL^{-1} Cu: $F_{(2, 37)} = 20.27$, $p < 0.001$, partial $\eta^2 = 0.52$; 2.5 μgL^{-1} Cu: $F_{(2, 37)} = 15.70$, $p < 0.001$, partial $\eta^2 = 0.46$).

However, when examining all pairwise comparisons, it was not possible to determine a time-concentration response of the gene (Fig. 5-3). Indeed, after 24h a slight but non-significant increase in gene expression was shown with increasing exposure concentration. At 96h, levels of expression were below the threshold of 1.5-fold. Finally, after 10 days, exposure to 1.25 μgL^{-1} Cu induced a significant upregulation, up to 5-fold, of HSP_{40} compared with unexposed snails and snails exposed to the highest concentration tested of 2.5 μgL^{-1} Cu. Indeed, at 2.5 μgL^{-1} Cu a significant downregulation (0.4 fold) of the gene was observed compared either to the control or to 1.25 μgL^{-1} Cu.

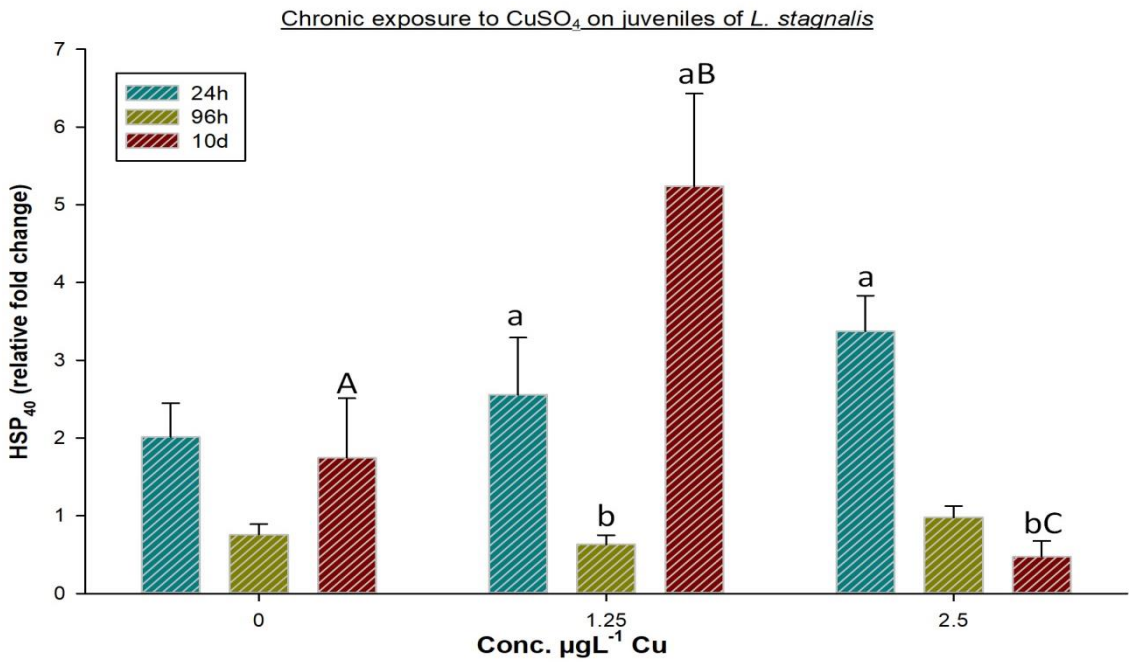

Figure 5-3 Expression levels of HSP_{40} gene in juvenile *L. stagnalis* after waterborne exposure of $CuSO_4$ for 10 days at different nominal concentrations (1.25 and 2.5 μgL^{-1}) of Cu. Data are means ± standard error. Different lowercase letters indicate significant differences ($p < 0.025$) within the same exposure concentration at different exposure time points; different capital letters indicate significant differences ($p < 0.025$) between different concentrations at the same exposure time point.

In contrast, exposure to pristine CuO NMs induced a significant expression of all the genes selected (Fig. 5-14).

A two-way ANOVA revealed a significant interaction between the increasing time of exposure and concentrations of tested pristine CuO NMs for all genes investigated, except HSP_{40}, as reported in Table 5-2.

Table 5-2 Results of Levene's test and Two-way ANOVA interaction results for the gene expression of the selected genes after exposure to increasing concentrations of pristine CuO NMs for 10 days.

Genes	Sig. Levene's test	Two-way ANOVA: Time*Conc. Interaction				
		df	*error*	*F*	*Sig.*	*partial* η^2
SOD	0.588	4	48	11.272	< 0.001	0.484
CAT	0.058	4	48	5.099	0.002	0.298
MT	0.210	4	47	3.551	0.013	0.232
HSP_{40}	0.016	4	37	1.828	0.144	0.165

Tukey's comparisons of the simple main effect for the 3 genes with significant interaction indicated that SOD was moderately significantly downregulated with increasing exposure time (fold < 0.04) at the two exposure concentration tested (250 μgL^{-1} Cu: $F_{(2, 48)} = 7.64$, $p = 0.001$, partial $\eta^2 = 0.24$; 500 μgL^{-1} Cu: $F_{(2,48)} = 22.98$, $p < 0.001$, partial $\eta^2 = 0.49$). Furthermore, the simple main effect assessment for exposure time showed a significant difference between concentrations at 24h and 10 days of exposure (24h: $F_{(2,48)} = 4.52$, $p = 0.02$, partial $\eta^2 = 0.16$; 10d: $F_{(2,48)} = 18.62$, $p < 0.001$,

partial η^2 = 0.44). Indeed, data at 24h showed an increase in the expression levels of SOD with increasing concentration and a significant downregulation of the gene after 10 days of exposure with increasing concentration of CuO NMs (Fig. 5-4 (1)).

In contrast, at 10 days, expression levels of CAT were significantly downregulated with exposure to pristine CuO NMs compared with the unexposed snails ($F_{(2, 48)}$ = 11.34, p < 0.001, partial η^2 = 0.32). However, no significant difference was revealed between the two exposure concentrations (Fig. 5-4 (2)).

A different modulation trend was observed, in contrast, for MT. Indeed, expression levels were modestly upregulated, up to 2-fold difference, when snails were exposed to pristine CuO NMs compared with unexposed snails (24h: $F_{(2, 47)}$ = 6.76, p = 0.003, partial η^2 = 0.22; 96h: $F_{(2, 47)}$ = 6.76, p < 0.001, partial η^2 = 0.39; 10d: $F_{(2, 47)}$ = 29.94, p < 0.001, partial η^2 = 0.56). However, no difference in the expression levels was revealed between the two concentrations, indicating that in this case the exposure time was the main factor driving the regulation of the gene (Fig. 5-4 (3) and C-1 in Appendix C).

Finally, two-way ANOVA revealed no significant interaction between concentration and time on the expression levels of HSP_{40} (Tab. 5-2), thus main effects of the two single factors (concentration and time) were evaluated. Results revealed a significant main effect of concentration and time of exposure on the modulation of HSP_{40} (concentration: $F_{(2,47)}$ = 8.80, p < 0.001; exposure time: $F_{(2,47)}$ = 6.25, p = 0.005). However, as shown in Fig. 5-4 (4), these significantly results are manly driven by the high expression (up to 3.5-fold difference) after 96h at 250 μgL^{-1} Cu of pristine CuO NMs; nevertheless, expression levels of the other time points are all above or close to the 1.5-fold set cut-off to consider a gene significantly express, indicating that the snails where negatively stressed by the exposure to pristine CuO NMs.

It is worth noting that taking in account only the nominal concentrations of Cu, a higher expression of the gene was expected compared to $CuSO_4$ given the higher concentration of nominal Cu tested. However, when comparing the two chemicals in terms of LC50, these results are unexpected. Indeed, $CuSO_4$ was tested at concentration equal to ½ LC50, 1.25 μgL^{-1} Cu, and ¼ LC50, 2.5 μgL^{-1} Cu, of $LC50_{96h}$. In contrast with pristine CuO NMs the concentration tested were equal to about ¼ LC50, 500 μgL^{-1} Cu, and 1/8 LC50, 250 μgL^{-1} Cu, of the $LC50_{96h}$ (see Chapter 2, Fig. 2-3 and 2-4), thus lower responses were predicted.

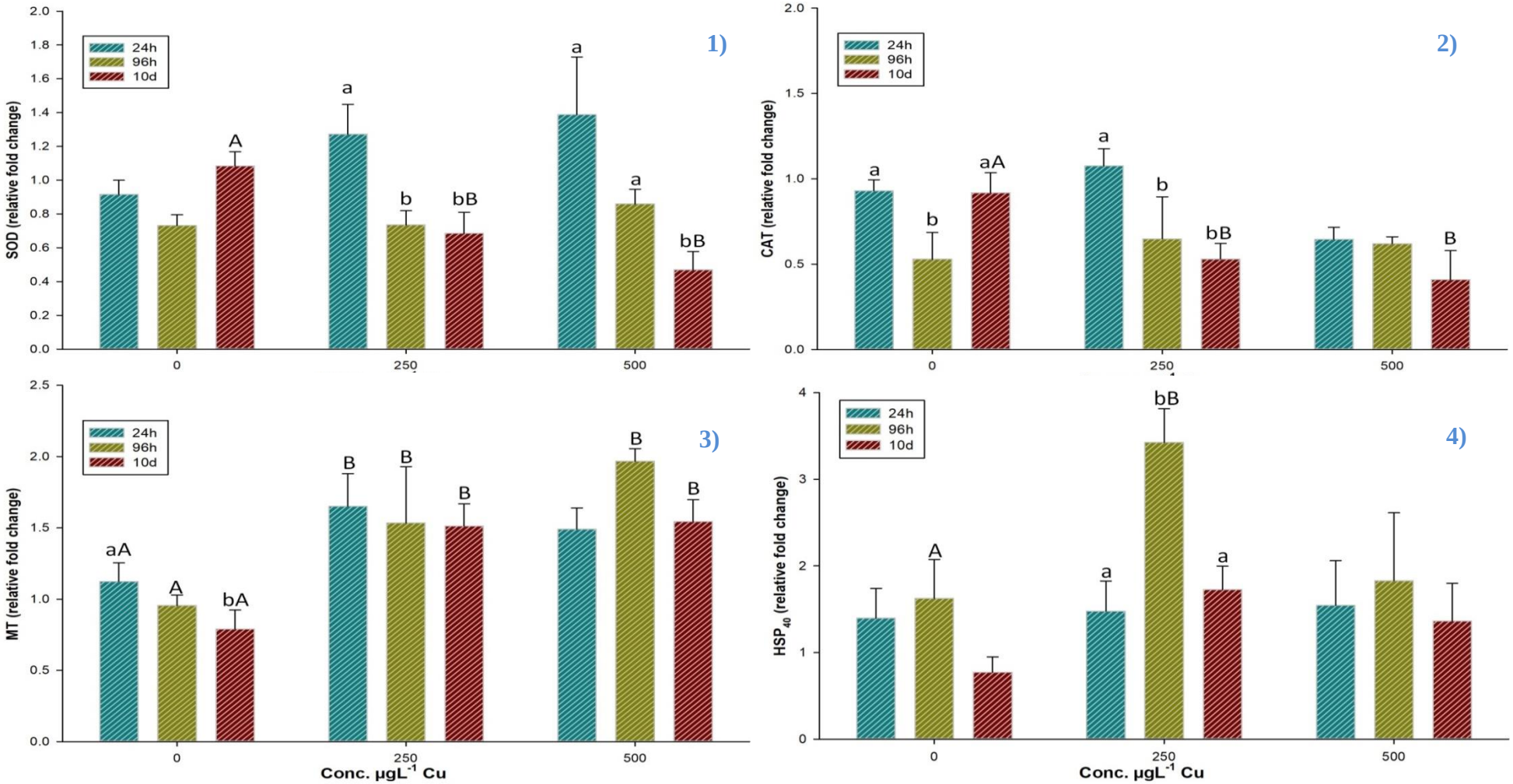

Figure 5-4 Expression levels of 4 selected genes in juveniles of *L. stagnalis* after waterborne exposure to pristine CuO NMs for 10 days at different nominal concentrations (250 and 500 µgL^{-1}) of Cu. 1) SOD; 2) CAT; 3) MT and 4) HSP_{40}. Data are means ± standard error. Different lowercase letters indicate significant differences ($p < 0.025$) within the same exposure concentrations between different exposure time points; different capital letters indicate significant differences ($p < 0.025$) between different concentrations at the same exposure time point.

Findings revealed a similar gene expression modulation, although eliciting a stronger response, for MT gene after exposure to increasing concentration of SbyD CuO-ASC NMs. Indeed, like for pristine CuO NMs, the expression of MT is significantly influenced by the interaction of the increasing concentration and exposure time, $F_{(4,47)}$ = 3.64, p = 0.01, partial η^2 = 0.2. Thus, simple main effects were calculated showing a significant effect of exposure time when snails were exposed to SbyD Cu-ASC NMs (30 µgL^{-1} Cu: $F_{(2,57)}$ = 10.65, p < 0.001, partial η^2 = 0.27; 60 µgL^{-1} Cu: $F_{(2,57)}$ = 11.77, p < 0.001, partial η^2 = 0.29); and a significantly different upregulation of the gene with increasing concentrations after 96h and 10d of exposure (96h: $F_{(2, 57)}$ = 6.26, p = 0.003, partial η^2 = 0.18; 10d: $F_{(2, 57)}$ = 20.30, p < 0.001, partial η^2 = 20.3) (Fig. 5-5).

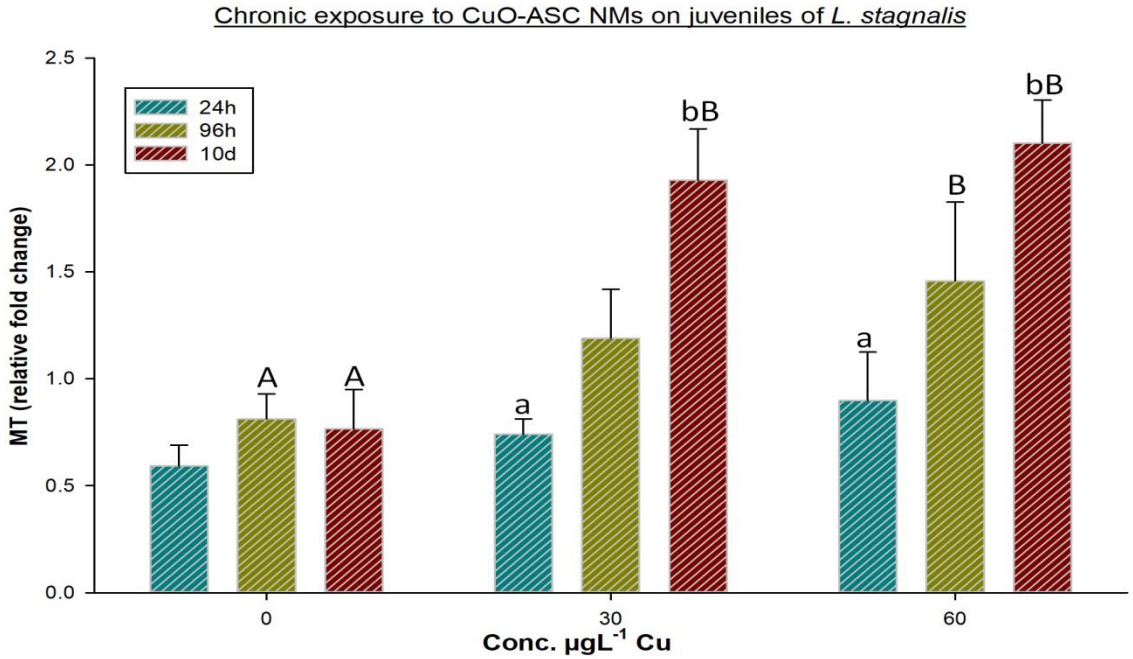

Figure 5-5 Expression levels of MT gene in juveniles of *L. stagnalis* after waterborne exposure to SbyD CuO-ASC NMs for 10 days at different nominal concentrations (30 and 60 µgL^{-1}) of Cu. Data are means ± standard error. Different lowercase letters indicate significant differences (p < 0.025) within the same exposure concentrations between different exposure time points; different capital letters indicate significant differences (p < 0.025) between different concentrations at the same exposure time point.

Furthermore, a two-way analysis of variance revealed a significant main effect of exposure time and exposure concentration on the expression HSP_{40} levels (concentration: $F_{(2, 57)}$ = 5.62, p < 0.006; exposure time: $F_{(2, 57)}$ = 11.04, p = 0.005), indicating that snails were subjected to stress due to the prolonged exposure to SbyD CuO NMs. The interaction effect was not significant, $F_{(4, 57)}$ = 1.27, p = 0.29. However, as for exposure to $CuSO_4$, no clear concentration or time exposure responses were observed. Indeed, at 24h and 10d a slight upregulation of the HSP_{40} was induced at 24h, reaching the maximum induction of 3-fold difference compared to 0h at the highest

concentration (60 µgL^{-1} Cu). However, at 96h no significant induction of the gene occurred.

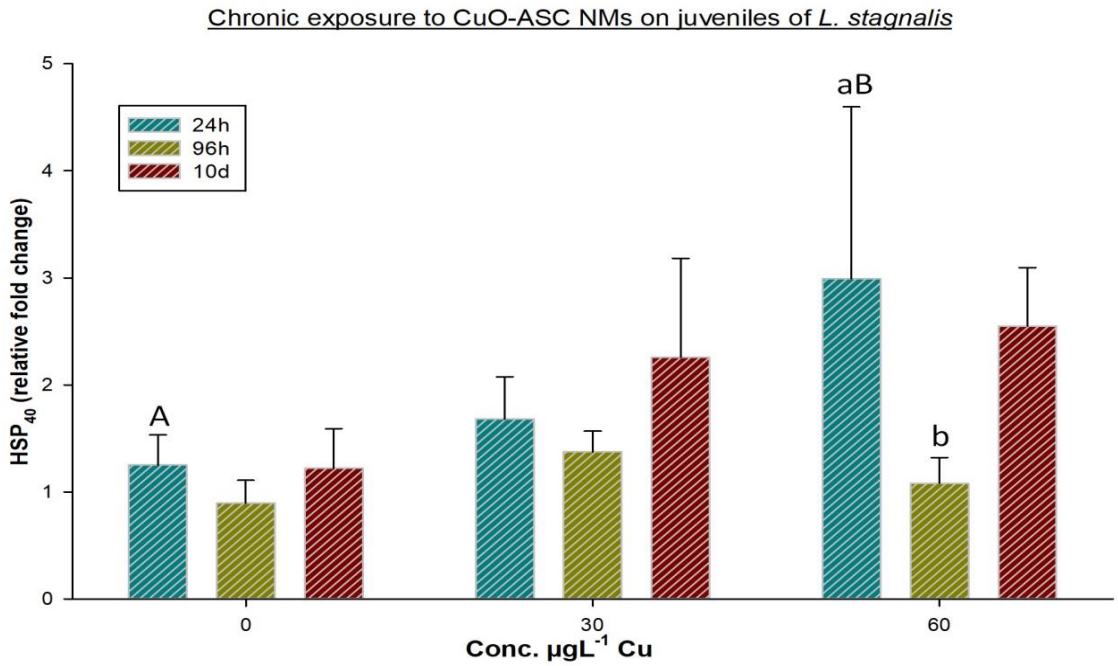

Figure 5-6 Expression levels of HSP_{40} gene in juveniles of *L. stagnalis* after waterborne exposure to SbyD CuO-ASC NMs for 10 days at different nominal concentrations (30 and 60 µgL^{-1}) of Cu. Data are means ± standard error. Different lowercase letters indicate significant differences (p < 0.025) within the same exposure concentrations between different exposure time points; different capital letters indicate significant differences (p < 0.025) between different concentrations at the same exposure time point.

Exposure to CuO_Acryl_FP and SbyD CuO-PVP NMs did not significantly induce the expression of any of the investigated genes (Fig. C-2 and C-3 in Appendix C).

5.3.2 Changes in gene expression in response to combined exposures to CuO NMs and heat shock

5.3.1.1 Effects after heat shock

Responses of the antioxidant and detoxification enzymes gene transcriptions were assessed, when snails were subjected to chronic exposure to CuO NMs, followed by a thermal shock (1h exposure at 30 °C).

As expected, overall the HSP_{40} gene, which is activated in response to stress and above all responds to increased temperature, was strongly upregulated after exposure to Cu in either form, ionic or nano, reaching expression levels up to 80-fold difference to the control at 0h (Fig. 5-7 and 5-8). However, as shown in the experiments without the thermal stress (see. 5.3.1.1), it was not possible to establish a clear concentration-time response relationship. Indeed, even when comparing unexposed snails (control) between all the experiments (Fig.5-7 and 5-8), expression levels were not homogenous after 10

days of exposure. At 24h exposure, thermal shock yielded an expression of HSP_{40} around 40-fold in all the experiments performed. In contrast, at 96h overall a downregulation of the gene was shown between 10 and 20-fold difference compared with time 0h. At 10 days, it is likely that the prolonged manipulation of the snails (*e.g.* change of water every three days) induced indiscriminate variations in the response (down or upregulation) of the snails to the thermal shock (Fig. 5-7 and 5-8).

Findings from exposure to ionic Cu as $CuSO_4$ (Fig. 5-7) revealed that the expression levels of HSP_{40} were significantly dependent on the interaction between concentration and time of exposure yielding a F ratio of $F_{(4, 50)} = 15.47$, $p < 0.001$, partial $\eta^2 = 0.55$). Furthermore, simple main effects for concentration and time of exposure revealed a significant effect of time exposure at all the concentrations tested (control: $F_{(2, 50)} = 8.80$, $p = 0.001$, partial $\eta^2 = 0.26$; 1.25 μgL^{-1} Cu: $F_{(2, 50)} = 16.98$, $p < 0.001$, partial $\eta^2 = 0.40$; 2.50 μgL^{-1} Cu: $F_{(2, 50)} = 21.4$, $p < 0.001$, partial $\eta^2 = 0.46$) and of exposure time after 24h and 10days (24h: $F_{(2, 50)} = 5.21$, $p = 0.009$, partial $\eta^2 = 0.17$; 10d: $F_{(2, 50)} = 24.36$, $p < 0.001$, partial $\eta^2 = 0.49$).

Overall, the same modulation pattern of HSP_{40} expression was shown compared with samples not subjected to further thermal stress (Fig. 5-3). Indeed, lower levels of expression were observed at 96h compared with the other two time points. At10d expression levels were upregulated at 1.25 μgL^{-1} Cu compared with the control and downregulated, instead, with the increase in exposure concentrations at 2.50 μgL^{-1} Cu (Fig. 5-7).

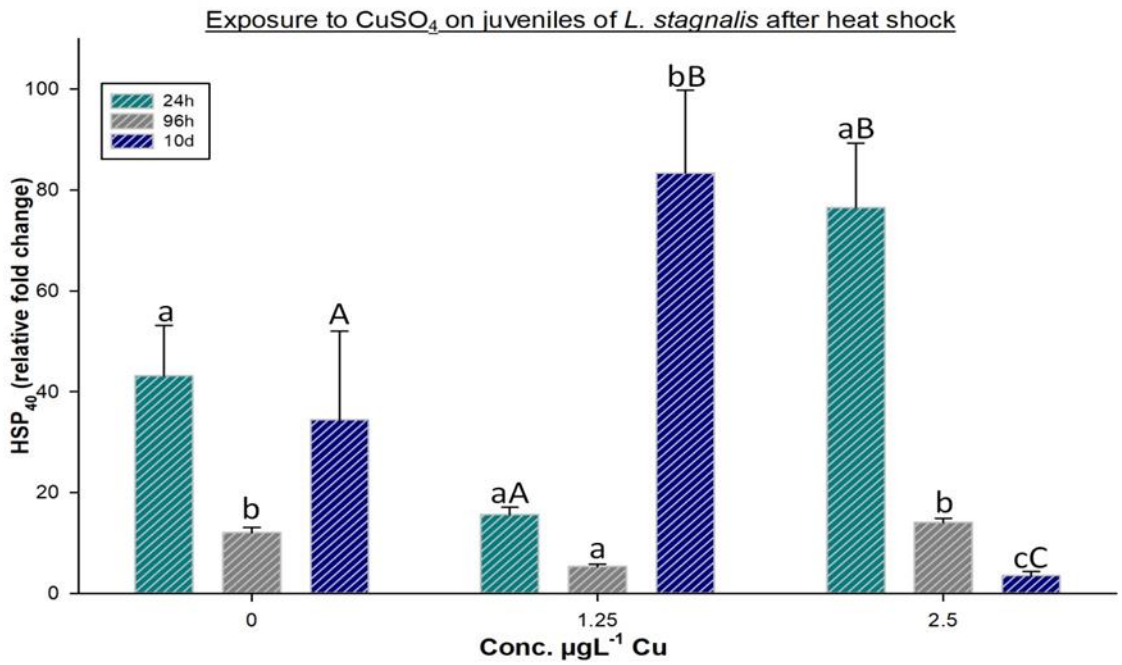

Figure 5-7 Expression levels of HSP_{40} gene in juveniles of *L. stagnalis* after waterborne exposure to ionic Cu as $CuSO_4$ for 10 days at different nominal concentrations (1.25 and 2.5 µgL^{-1}) of Cu. Data are means ± standard error. Different lowercase letters indicate significant differences ($p < 0.025$) within the same exposure concentrations between different exposure time points; different capital letters indicate significant differences ($p < 0.025$) between different concentrations at the same exposure time point.

Transcription levels of HSP_{40} were significantly affected by the interaction of the two factors, increasing concentration and time, only for the snails exposed to SbyD CuO-ASC NMs (Tab. 5-3); exposure to the other three NMs, instead, yielded only a significant main effect on the expression levels of HSP_{40} only with the increase in exposure time within each treatment (pristine CuO NMs, $F_{(2, 36)} = 29.72$, $p < 0.001$; CuO_Acryl_FP, $F_{(2, 31)} = 7.34$, $p = 0.002$; SbyD CuO-PVP, $F_{(2, 35)} = 18.17$, $p < 0.001$).

Table 5-3 Results of Levene's test and Two-way ANOVA interaction for the gene expression of HSP_{40} after exposure to increasing concentrations of different NMs for 10 days.

NMs	Sig. Levene's test	Two-way ANOVA Time*Conc. Interaction				
		df	*error*	*F*	*Sig.*	*partial* η^2
Pristine CuO	0.168	4	36	0.668	0.618	n.d.
CuO_Acryl_FP NMs	0.131	6	31	2.017	0.093	n.d.
SbyD CuO-ASC NMs	0.509	4	30	6.432	0.01	0.462
SbyD CuO-PVP NMs	0.948	4	35	0.978	0.432	n.d.

Among all the different NMs and materials tested, it is worth noticing the effect of exposure to SbyD CuO-ASC on the expression of HSP_{40}. Indeed, this is the only case in which a clear modulation pattern of the gene is shown, indicating a strong

downregulation of the gene with increasing exposure concentration and exposure time. A stronger response was determined after 10 days of exposure between unexposed and exposed snails, showing a decrease in gene expression of about 40-fold difference between control snails and those exposed to 30 and 60 μgL^{-1} Cu of SbyD CuO-ASC NMs ($F_{(2, 30)} = 13.83$, $p < 0.001$, partial $\eta^2 = 0.48$).

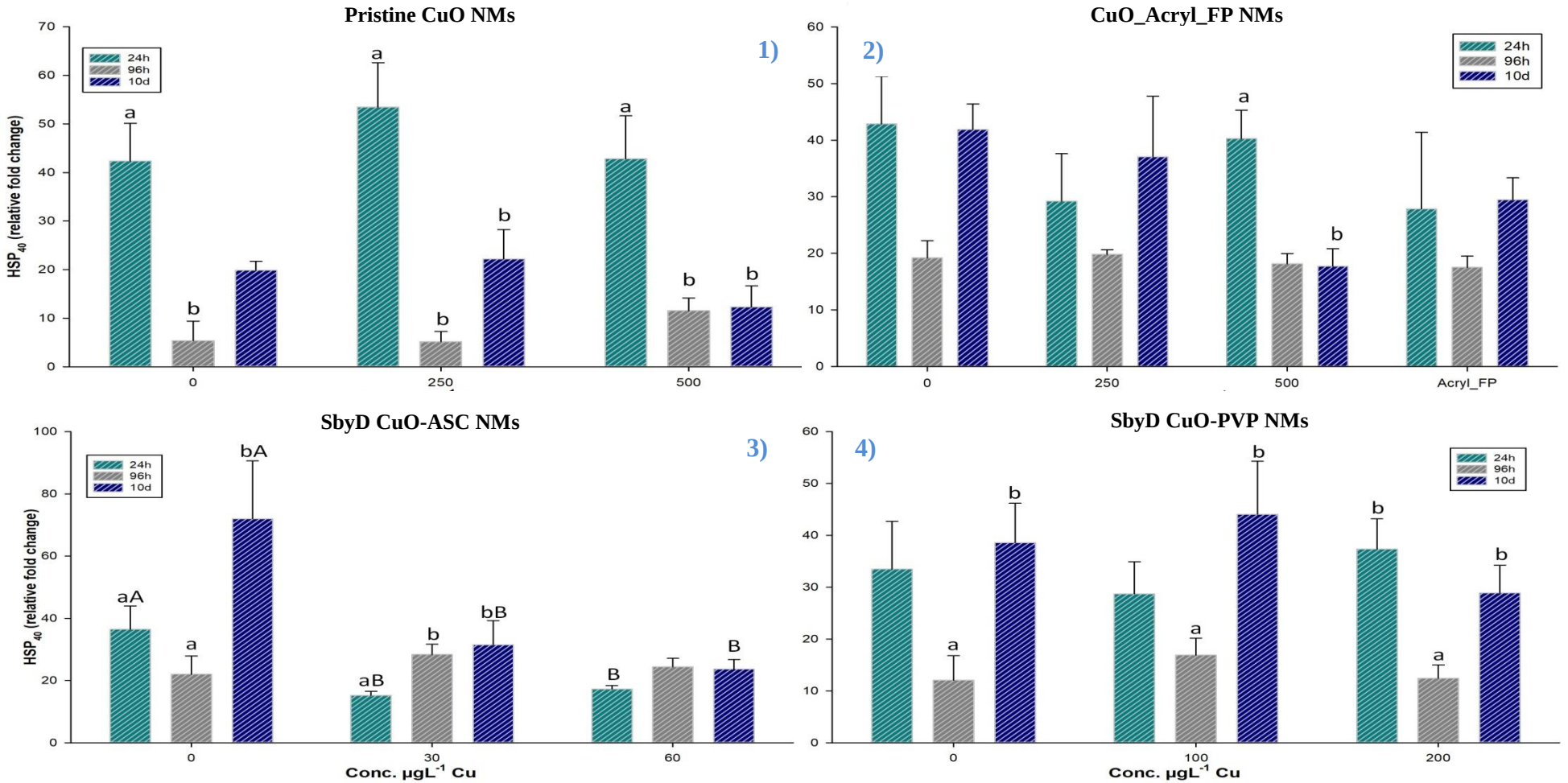

Figure 5-8 Expression levels of HSP_{40} gene in juveniles of *L. stagnalis* after 1 h heat shock (30 °C) and waterborne exposure to 4 different CuO NMs for 10 days at two different nominal concentrations corresponding to the ¼ and 1/8 of their $LC50_{96h}$. Data are means ± standard error. Different lowercase letters indicate significant differences ($p < 0.025$) within the same exposure concentrations between different exposure time points; different capital letters indicate significant differences ($p < 0.025$) between different concentrations at the same exposure time point.

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Furthermore, in contrast with the previous experiments without the heat shock, findings revealed that the additional thermal stress activated the transcription of both antioxidant enzymes investigated, SOD and CAT, in juvenile's snails when exposed to $CuSO_4$, with a stronger response, up to 2.5-fold, of SOD for unexposed snails after 10 days. Both genes were significantly affected by the interaction of increasing concentration and time of exposure, yielding an F ratio of $F_{(4, 51)} = 2.80$, $p = 0.03$, partial $\eta^2 = 0.18$ and $F_{(4, 51)} = 3.9$, $p = 0.008$, partial $\eta^2 = 0.23$, respectively, for SOD and CAT.

In particular, expression of SOD was significantly upregulated only at 24h with increasing concentration of $CuSO_4$ ($F_{(2, 51)} = 8.02$, $p = 0.001$, partial $\eta^2 = 0.24$) (Fig. 5-9 (left)), instead, CAT was negatively regulated at 96h ($F_{(2, 51)} = 6.11$; $p = 0.004$, partial $\eta^2 = 0.19$) (Fig. 5-9 (right)) with increasing concentration of Cu and, remaining almost unvaried and within the threshold of1.5/0.5-foldd at 10d of exposure.

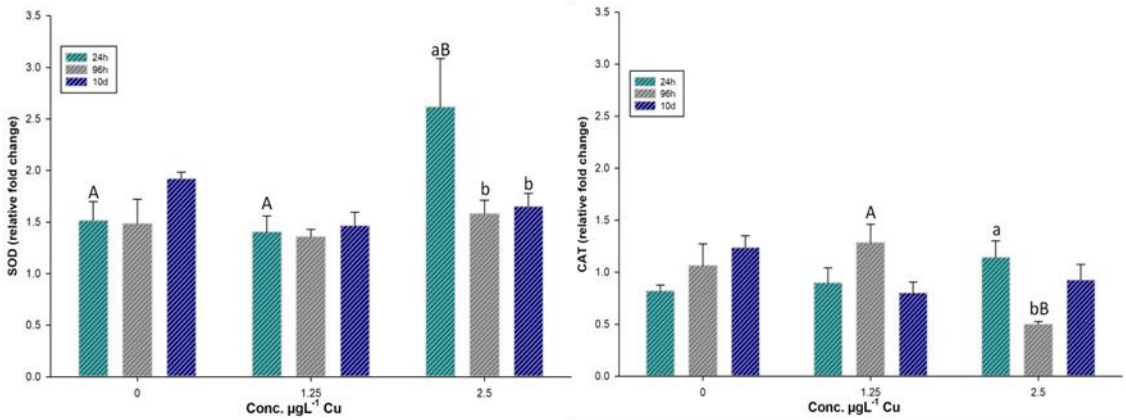

Figure 5-9 Expression levels of SOD (left) and CAT (right) genes in juveniles of *L. stagnalis* after 1 h heat shock (30 °C) and waterborne exposure to ionic Cu as $CuSO_4$ for 10 days at different nominal concentrations (1.25 and 2.5 µgL^{-1}) of Cu. Data are means ± standard error. Significant differences ($p < 0.025$) are indicated by lowercase letters within the same exposure concentration at different exposure time points and by different capital letters between different concentrations at the same exposure time point.

Exposure to pristine CuO NMs, instead, induced a stronger downregulation of CAT compared to $CuSO_4$. Indeed, in agreement with the previous experiment without heat shock, expression levels of CAT were modestly significant downregulated (0.49-fold difference to 0h), when snails were exposed to the two concentrations of pristine CuO NMs tested, 250 and 500 µgL^{-1}, yielding a F ratio of $F_{(2, 36)} = 10.74$, $p < 0.001$ (Fig. 5-10). Similarly, no difference in the expression of SOD was revealed compared to the experiment without heat shock was demonstrated, indicating a slight downregulation of the gene with increasing of exposure time within the same concentration of exposure (Fig. 5-10). In contrast, a total opposite induction was shown for MT, which in agreement with the previous experiment, was upregulated up to 2-fold difference to the

control with increasing of concentration exposure ($F_{(2, 36)}$ = 11.86, p < 0.001) (Fig. 5-11).

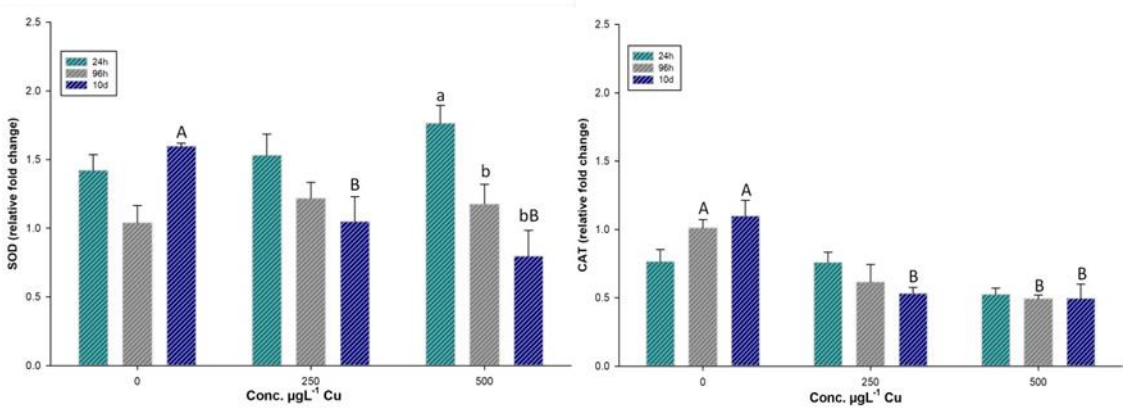

Figure 5-10 Expression levels of SOD (left) and CAT (right) genes in juveniles of *L. stagnalis* after 1 h heat shock (30 °C) and waterborne exposure to Cu as pristine CuO NMs for 10 days at different nominal concentrations (250 and 500 µgL^{-1}) of Cu. Data are means ± standard error. Significant differences (p < 0.025) are indicated by lowercase letters within the same exposure concentration between different exposure time points and by different capital letters between different concentrations at the same exposure time point.

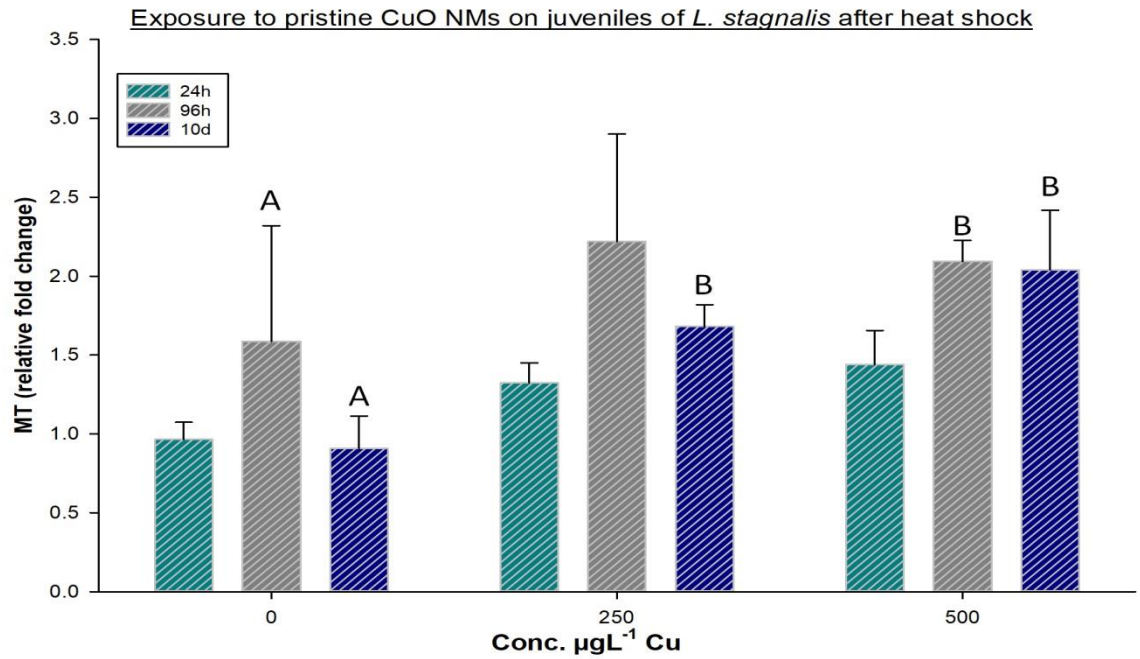

Figure 5-11 Expression levels of MT gene in juveniles of *L. stagnalis* after 1 h heat shock (30 °C) and waterborne exposure to Cu as pristine CuO NMs for 10 days at different nominal concentrations (250 and 500 µgL^{-1}) of Cu. Data are means ± standard error. Significant differences (p < 0.025) are indicated by lowercase letters within the same exposure concentration between different exposure time points and by different capital letters between different concentrations at the same exposure time point.

Progressive upregulation of MT was determined also in snails exposed to SbyD CuO-ASC with increasing concentration and time of exposure ($F_{(2, 38)}$ = 16.85, p < 0.001) (Fig. 5-12). Levels of MT expression did not change with the addition thermal stress, instead SOD was positively regulated in control snails at 10 days of exposure (2.5 fold), returning then to control levels when snails were exposed to the NMs ($F_{(2, 36)}$ = 4.26, p = 0.02) (Fig. 5-13).

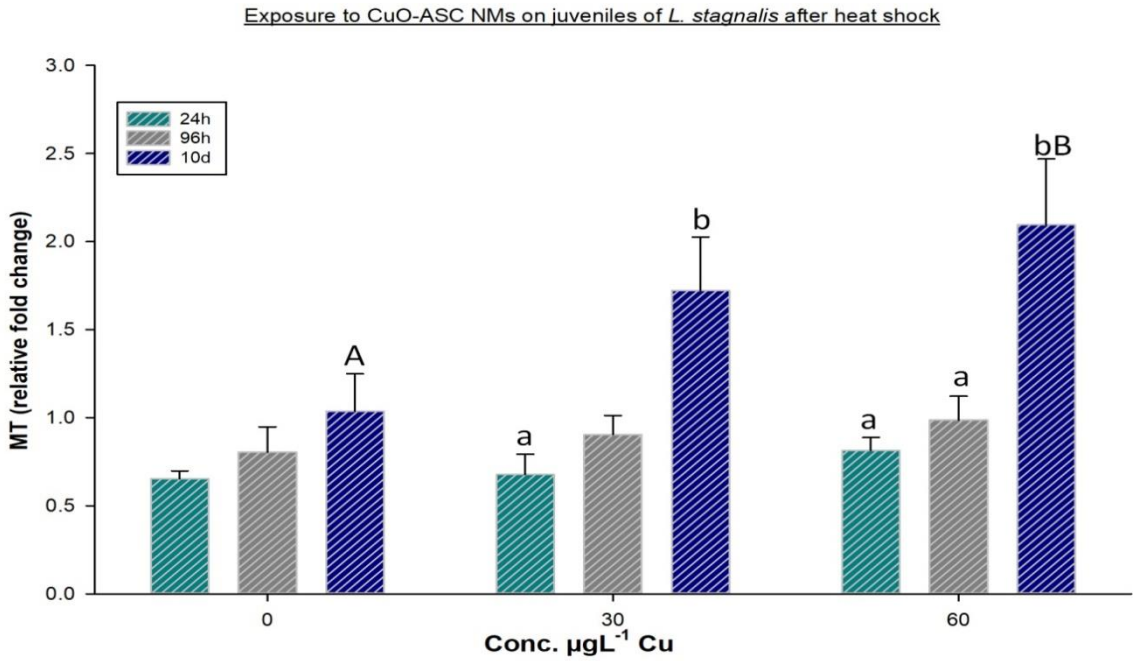

Figure 5-12 Expression levels of MT gene in juveniles of *L. stagnalis* after 1 h heat shock (30 °C) and waterborne exposure to Cu as SbyD CuO-ASC NMs for 10 days at different nominal concentrations (30 and 60 µgL^{-1}) of Cu. Data are means ± standard error. Significant differences (p < 0.025) are indicated by lowercase letters within the same exposure concentration between different exposure time points and by different capital letters between different concentrations at the same exposure time point.

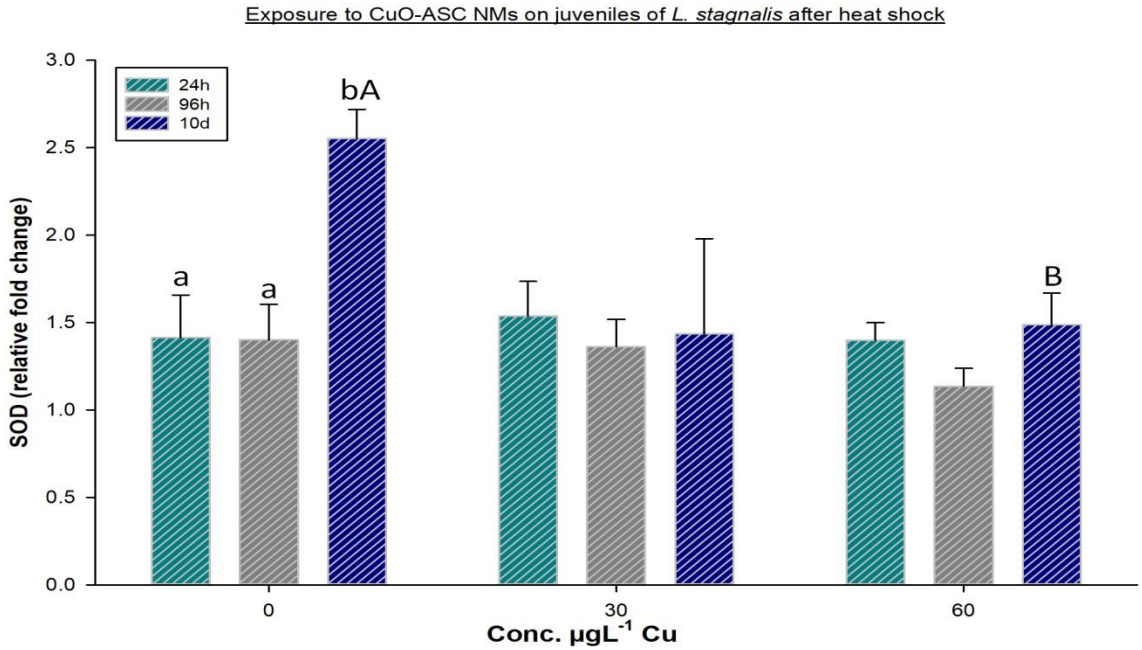

Figure 5-13 Expression levels of MT gene in juveniles of *L. stagnalis* after 1 h heat shock (30 °C) and waterborne exposure to Cu as SbyD CuO-ASC NMs for 10 days at different nominal concentrations (30 and 60 µgL^{-1}) of Cu. Data are means ± standard error. Significant differences (p < 0.025) are indicated by lowercase letters within the same exposure concentration at different exposure time points and by different capital letters between different concentrations at the same exposure time point.

Finally, in contrast with the previous test (Appendix C, Fig. C-3), thermal stress induced significantly, yet modest, expression of SOD and MT in snails exposed to CuO_Acryl_FP. In particular, MT and SOD were significantly, respectively, up (Fig. 5-

14) and downregulated (Fig. 5-15) with increasing of exposure time within the same treatment ($Fs_{(2, 32)}$ = 11.44 (SOD)/ 9.99 (MT), $p < 0.001$).

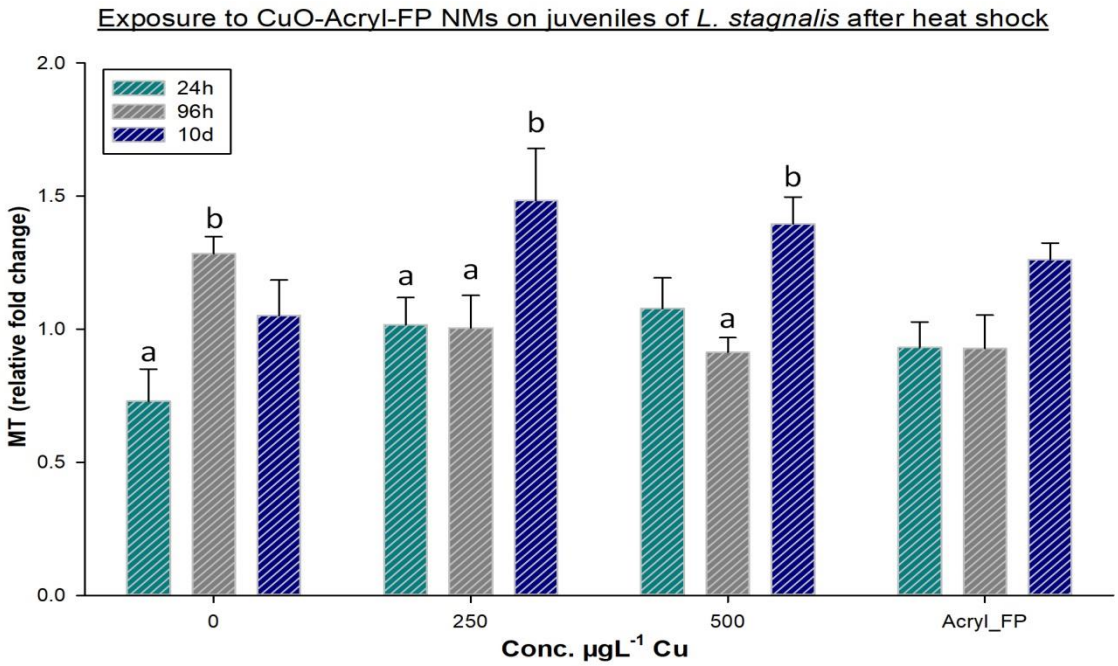

Figure 5-14 Expression levels of MT gene in juveniles of *L. stagnalis* after 1 h heat shock (30 °C) and waterborne exposure to Cu as CuO_Acryl_FP NMs for 10 days at different nominal concentrations (250 and 500 µgL^{-1}) of Cu. Data are means ± standard error. Significant differences ($p < 0.025$) are indicated by lowercase letters within the same exposure concentration between different exposure time points and by different capital letters between different concentrations at the same exposure time point.

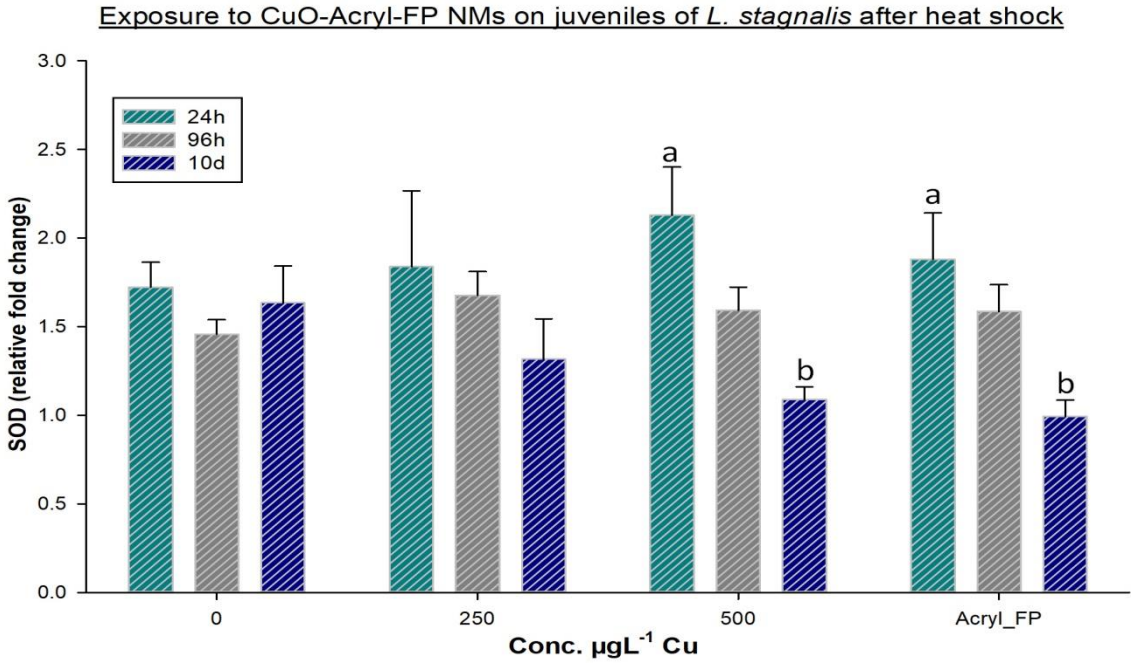

Figure 5-15 Expression levels of MT gene in juveniles of *L. stagnalis* after 1 h heat shock (30 °C) and waterborne exposure to Cu as CuO_Acryl_FP NMs for 10 days at different nominal concentrations (250 and 500 µgL^{-1}) of Cu. Data are means ± standard error. Significant differences ($p < 0.025$) are indicated by lowercase letters within the same exposure concentration between different exposure time points and by different capital letters between different concentrations at the same exposure time point.

5.3.1.2 Effect after 4 hours recovery from heat shock

After 4h recovery from the heat shock, snails' modulation of the detoxification and antioxidant system enzymes was evaluated to determine a time profile of the selected genes.

Overall, findings revealed a reduction of around 1 order of magnitude in the expression levels of HSP_{40}, remaining, however, above the control threshold of 1.5-fold, in snails exposed to Cu in either forms. This result suggested that after 4 hours of recovery from the thermal stress, protein folding and degradation still occurred necessitating the action of HSPs in preventing further damage.

After 4 hours recovery from the heat shock, snails' exposure to ionic Cu induced significant levels of HSP_{40} (Fig. 5-16), which were however strongly downregulated (≈ 1 order of magnitude) compared with samples taken just after heat shock (Fig. 5-6) and which went almost back to the same levels before heat shock (Fig. 5-2).

Overall, a reduction of 1 order of magnitude in the transcription levels of HSP_{40} after 4 h recovery from the heat shock, was recorded for ionic Cu and all the CuO NMs (Fig. 5-16 and 5-17), with the exception of pristine CuO NMs (Fig. 5-7 and 5-17).

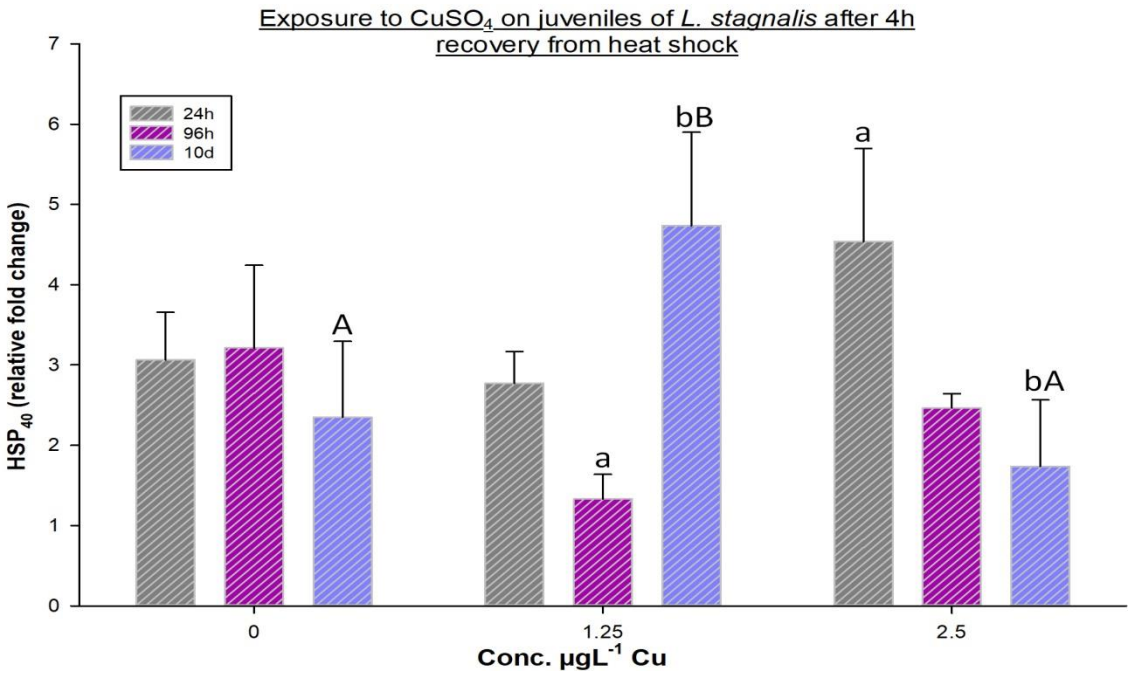

Figure 5-16 Expression levels of HSP_{40} gene in juveniles of *L. stagnalis* after 1 h heat shock (30 °C) and waterborne exposure to ionic Cu as $CuSO_4$ NMs for 10 days at different nominal concentrations (1.25 and 2.5 μgL^{-1}) of Cu. The data are means ± standard error. Different lowercase letters indicate significant differences ($p < 0.025$) within the same exposure concentration between different exposure time points; different capital letters indicate significant differences ($p < 0.025$) between different concentrations at the same exposure time point.

Differently from the other CuO NMs, when snails where exposed to pristine CuO NMs, after 4 hours of recovery from the heat shock, transcription of HSP_{40} followed a different modulation patter. Indeed, after 24h of exposure a strongly upregulation of the

gene was recorded with increasing concentration ($F_{(2,37)} = 8.80$, $p < 0.001$), maintaining, at the highest concentration tested of 500 μgL^{-1} Cu, still very high levels of HSP_{40} which were only reduced 3 times compared with samples taken just after the heat shock (Fig. 5-17). Furthermore, at 10 days, a same concentration dependent upregulation, although not significant, of the gene was shown after 10 days contrary to the previous exposure conditions with, or before, heat shock where levels of expression did not change with increasing concentration (see Appendix C; Fig. C-5).

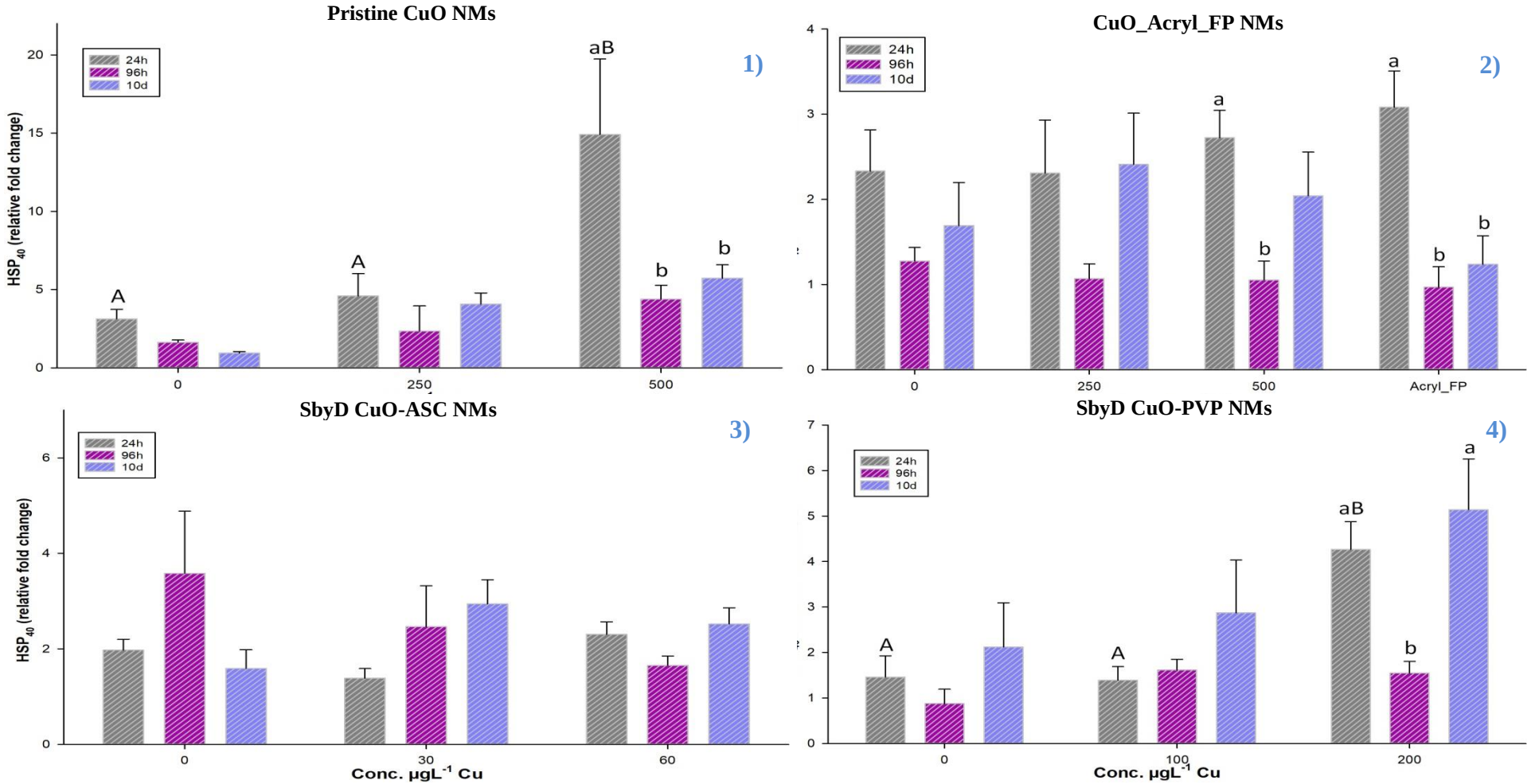

Figure 5-17 Expression levels of HSP_{40} gene in juveniles of *L. stagnalis* after 4h recovery from the heat shock and waterborne exposure to 4 different CuO NMs for 10 days at two different nominal concentrations corresponding to the ¼ and 1/8 of their $LC50_{96h}$. Data are means ± standard error. Different lowercase letters indicate significant differences ($p < 0.025$) within the same exposure concentration between different exposure time points; different capital letters indicate significant differences ($p < 0.025$) between different concentrations at the same exposure time point.

The second most sensitive gene after recovery form the thermal shock was MT, which was significantly expressed in experiments using all the CuO NMs, except of SbyD CuO-PVP NMs. For all the NMs, gene expression levels remained within the range of expression showed after the heat shock (Fig, 5-18), however after 96h exposure to pristine CuO NMs, a simple main effect revealed a significant downregulation of the gene with the increase in concentration ($F_{(2, 38)} = 9.26$, $p = 0.001$, partial $\eta^2 = 0.33$).

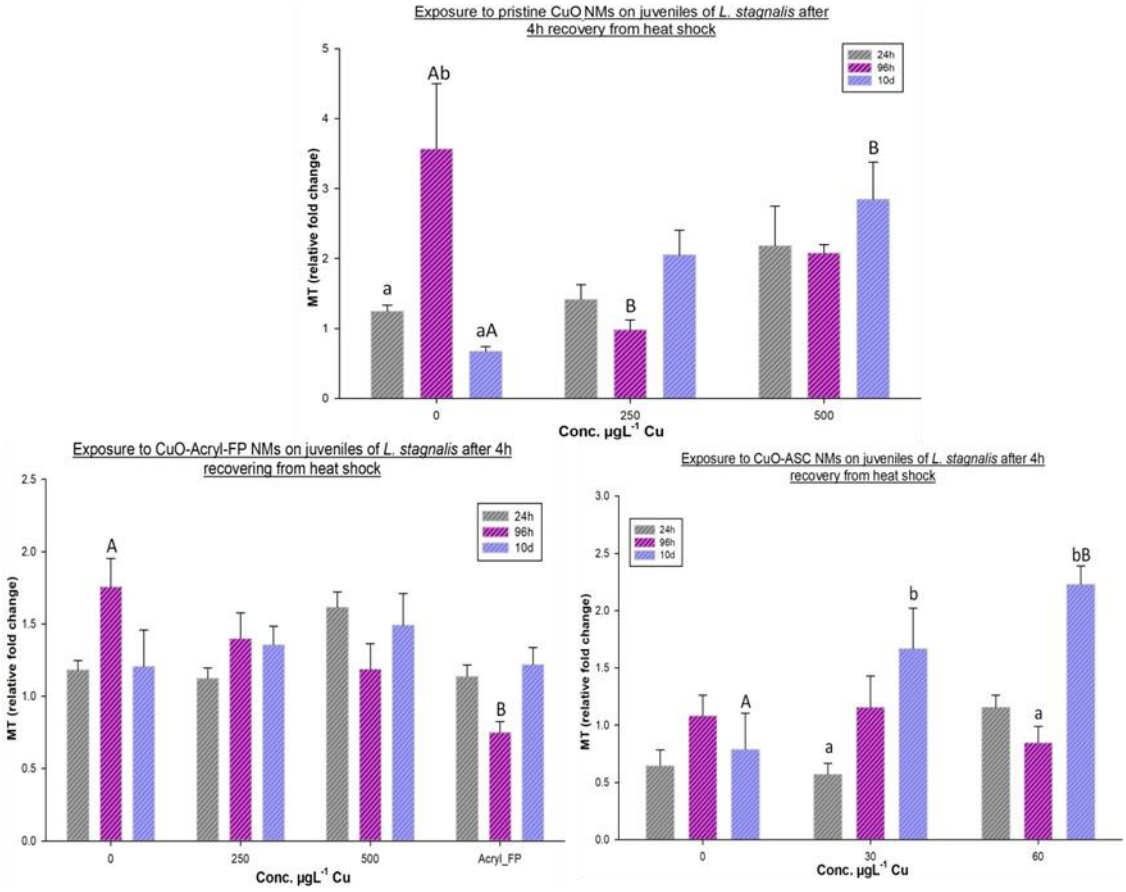

Figure 5-18 Expression levels of MT gene in juveniles of *L. stagnalis* after 4 h recovery from the heat shock (30 °C) and waterborne exposure to 3 different CuO NMs for 10 days at increasing nominal Cu concentrations. Data are means ± standard error. Different lowercase letters indicate significant differences (p < 0.025) within the same exposure concentration between different time points; different capital letters indicate significant differences (p < 0.025) between different concentrations at the same exposure time point.

5.4 Discussion

The main aim of this research study was to evaluate the modulation of antioxidant and detoxification enzymes in response to sub-chronic exposure of juveniles of *L. stagnalis* to different CuO NMs. Furthermore, snails were subjected to additional thermal shock, at specific time points, to investigate the snails' response to environmental stresses during sub-chronic exposure to NMs. To achieve this aim, the study involved the assessment of gene expression levels of SOD, CAT, MT and HSP_{40} enzymes after 24h, 96h and 10 days of exposure to the different CuO NMs with or without thermal stress.

5.4.1 Changes in gene expression in response to sub-chronic exposure studies of CuO NMs

Exposure to the ionic control of Cu^{2+} as $CuSO_4$ did not significantly changed the transcript levels of antioxidant enzymes or MT. At sublethal concentrations, only HSP_{40} was significantly expressed (Fig. 5-2 and 5-3), indicating, a general sign of stress due to exposure to Cu ions. A previous study (Atli and Grosell 2016), measuring the enzymatic activity of CAT and SOD in adults of *L. stagnalis*, showed high CAT activity, depending on the tissue examined at concentrations as low as 2 μgL^{-1} Cu of $CuSO_4$ after 96h of exposure. It is likely that the different responses found in this study can be explained by the method used to determine response to oxidative stress.

For instance, Woo et al. (2013) evaluating the expressions of oxidative stress-related genes and antioxidant enzyme activities in *M. galloprovincialis* in hypoxia, determined that changes in gene expression do not always correspond with enzyme activities. Indeed, after 24h and 48h of hypoxia, CAT activity significantly decreased with oxygen depletion, while no significant expression of the CAT gene was measured. The authors attributed this discrepancy to the time-profile of the gene response, suggesting that at that time point the peak of response of the gene had already occurred, and their subsequent triggering of protein expression or activity, changed depending on the intensity of the stress (Woo et al. 2013).

A similar response could have occurred in this study, where it is likely that a stronger response of the antioxidant enzymes happened within the first 24 hours of exposure. Indeed, the high levels of HSP_{40} found when snails were exposed to $CuSO_4$ corroborate this hypothesis. HSP_{40}'s main functions is to act to prevent the aggregation and degradation of protein due to heavy metal-induced oxidative stress (Kim et al. 2018), however, the current literature presents conflicting results on the role of HSPs in the prevention of oxidative stress, which appears to be species and exposure condition specific.

For example, Zhao et al (2017) exposing cucumber plants to a $Cu(OH)_2$ nano-pesticide for 3 weeks, found a negative correlation between oxidative stress and expression of HSP_{40}. Gene expression analyses showed that the increase in oxidative stress induced a decrease in expression levels of HSP_{40} in a concentration-dependent trend. Contrasting results were found by Kim et al. (2018) analysing the transcriptional profile, after 8h exposure to Cu (25 μgL^{-1}), of the antioxidant defence system and HSPs families on the marine ciliate *Euplotes crassus.* Findings showed a significantly high upregulation of

HSP_{40} due to an increase in oxidative stress determined by the upregulation of enzymes of the antioxidant system such as GSH-related genes (GST theta, GPx and GR) and SOD (Mn-SOD and Cu/Zn-SOD).

In this research study, further indication of the contribution of HSP_{40} in the modulation of antioxidant defence system of *L. stagnalis* can be found when snails were exposed to pristine CuO NMs. In this instance, all 4 genes selected, SOD, CAT, MT and $HSP_{40,}$ were significantly expressed (below or above the 0.5/1.5-fold threshold and $p < 0.025$), indicating a correspondence between the downregulation of SOD and CAT with increasing concentration and exposure time to CuO NMs, and upregulation of MT and higher response in fold differences in HSP_{40} (Fig. 5-4). These results are in accordance with previous studies (Griffitt et al. 2007, Ali and Ali 2015, Siddiqui et al. 2015) where a higher ROS formation due to CuO NMs compared to $CuSO_4$ was demonstrated, suggesting that CuO NMs effects cannot be solely attributed to dissolution.

SOD is the first defence response to ROS catalysing the dismutation of superoxide anion radical into water and hydrogen peroxide, which CAT then reduces to water and oxygen for elimination of ROS (Atli and Grosell 2016) and thus protecting the organisms from oxidative stress. In fact, in the present study, after exposure to pristine CuO NMs, at 24h, SOD gene expression increased with increasing concentration, and CAT increased at 250 μgL^{-1} Cu compared to the control, however it was then downregulated at the highest concentration tested of 500 μgL^{-1} Cu (Fig. 5-4). At 96h and 10d both genes were downregulated, suggesting an overload of the antioxidant system, determining thus an increase in HSP_{40} and MT production, which acted in preventing further protein degradation and active cell detoxification from Cu (Fig. 5-4) (Doganlar and Doganlar 2015).

Significant expression of MT and HSP_{40} were also found in snails exposed to SbyD CuO-ASC NMs (Fig. 5-5 and 5-6), however with a clearer pattern compared to pristine CuO NMs. Indeed, a marked upregulation of the two genes with increasing concentration and time of exposure was observed in this case. However, as for $CuSO_4$ no significant expression of the gene concerning specifically the antioxidant system was determined. It is worth noting that in this case, at the concentrations tested 30 and 60 μgL^{-1} Cu, around 30% mortality of the population was observed, and thus indicating a likely pro-oxidant function of ascorbate potentially contributing and increasing the toxicity of the core of CuO NMs, as indicated in the study described in Chapter 2.

Finally, no significant expression of any of the genes was measured when snails were exposed to either SbyD CuO-PVP NMs or CuO_Acryl_FP.

Thus, results suggest that the adverse effects of pristine CuO NMs were likely mitigated or eliminated by incorporation of the PVP surface coatings, likely due to the reduction of their agglomeration and dissolution on OECD medium (HDD 1159 ± 256, $Cu_{dissolved}/CuO_{total}$ (%) 0.1). Furthermore, the change in charge (ζ potential=+1.6 ± 0.3), from negative to positive, although small. compared to the core of pristine CuO NMs (see chapter 2, section 2.3.1) might have modified their intracellular distribution and production of ROS resulting thus in a lower toxicity (Sharifi et al. 2012).

Finally, the non-lethal toxicity as well as the absence of any molecular response due to exposure to CuO_Acryl_FP found in this study highlights the need for more studies assessing the risk of different forms of NMs more representative of real exposure scenarios, such as NMs embedded in their matrix, rather than solely their pristine form.

5.4.2 Changes in gene expression in response to combined exposures to CuO NMs and heat shock

Different cellular mechanisms, such as increasing heat shock and antioxidant system proteins, are involved in the maintenance of physiological homeostasis and resilience to thermal stress in an organism (Koopman et al. 2016, Coggins et al. 2017, Khomich et al. 2017). These mechanisms allow organisms to overcome negative consequences of thermal stress, including the accumulation of free radicals, protein degradation and energy depletion. However, the response to thermal stress depends on the environmental condition of their control habitat. For example, Gleason and Burton (2015) investigating the transcriptome response to thermal stress in geographically separated populations of the marine snail *Chlorostoma funebralis*; found a greater response in the gene expression of HSPs in the northern population, no used to frequent fluctuations in environmental temperature, compared to the two southern populations more used to temperature changes. Furthermore, several molecular chaperones and antioxidant genes that were not differentially expressed in southern populations showed higher expression under control conditions compared to northern populations, suggesting the development of oxidative stress and activation of protective nonspecific mechanisms of stress adaptation.

Results obtained in the present study strongly suggested the involvement of both constitutive and inducible heat shock proteins along with oxidative stress response in *L. stagnalis* to combined thermal stress and CuO NMs, however the extent of the contribution of the two factors it is not straightforward.

Overall, it appeared that a significant interaction occurred between increasing concentration and time of exposure, yielding a stronger upregulation of HSP_{40} after the heat shock by up to 80-fold (Fig. 5-7 and 5-8 (3), Tab. 5-3) compared to the control at 0h.

However, when evaluating only the unexposed snails in the different experiments, no clear time response relationship could be determined. Indeed, at 24h, after heat shock, a marked upregulation of $HSP_{40,}$ around 40-fold compared to the control was revealed, indicating an adaptation or compensatory reaction to the thermal stress, which at 96h was less strong (≈ 20-fold). It is therefore likely, that the clear response at 24h was the result of an additive stress due to the manipulation, during the experiment set up, of the snails within a short amount of time. Snails at 96h might had, instead, time to overcome the manipulation stress, since no further water changes were performed from the start of the experiment. This is further confirmed from the results gathered at 10 days, when gene expression levels of HSP_{40} of unexposed snails subjected to heat shock after 10 days of manipulation (water change every 3 days) were different in each of the experiments (Fig. 5-7 and 5-8).

This response pattern is also seen in snails exposed to the different CuO NMs and Cu ions. Indeed, when no significant interaction between concentration and time of exposure was shown, no significant changes in the expression of HSP_{40} were seen with increasing exposure concentration compared with the control. An arbitrary upregulation of the gene was instead seen when snails were exposed to 1.25 μgL^{-1} Cu of $CuSO_4$ after 10 days and to 2.5 μgL^{-1} Cu after 24 of exposure. In contrast, a significant downregulation of the gene was shown when snails were exposed to SbyD CuO-ASC NMs (Fig. 5-7 and 5-8).

A high increase of HSPs (HSP_{70}) was observed also by Axenov-Gribanov et al. (2015) exposing adults of eastern Siberian populations of *L. stagnalis* to a gradually increase in temperature from 6 to 30 °C, confirming the thermal sensitivity of this species. Furthermore, these authors showed a significant positive activation of the antioxidant enzymes as peroxidase and glutathione S-transferase and a negative activity of CAT with increasing temperature.

Accordingly, in this study, after the thermal stress, coupled with the marked and expected expression of HSP_{40}, genes responsible for the antioxidant system were significantly expressed. This trend indicated that snails were subjected to an enhanced and most likely extended pro-oxidant challenge, which resulted at the sampling time, in the determination of significant levels of the genes (Axenov-Gribanov et al. 2015,

Gleason and Burton 2015). SOD was significantly expressed following exposure to all the NMs, except for SbyD CuO-PVP. MT was also significantly upregulated when snails were exposed to all the NMs; however, no strong changes in the expression levels were seen with increased temperature when the gene was also expressed in absence of thermal stress (Fig. 5-4(3), 5-5 and 5-18).

The hypothesis of a heightened oxidative stress due to thermal shock is further corroborated by the snails exposed to CuO_Acryl_FP. Indeed, after additive exposure to a thermal shock, snails exhibited a significant upregulation of HSP_{40} and MT, and downregulation of SOD, which were not expressed with merely exposure to the NM for 10 days (Fig. 5-8, 5-14 and 5-15). In particular, data showed that up to 24h, SOD acted as the first line of defence against ROS by catalysing the dismutation of $^{\bullet}O_2^-$ to H_2O_2 (Zhao et al. 2016, Zhao et al. 2017), determining an increase, although not significant, in expression of the gene with increased exposure concentration (Fig. 5-14). After 24h, the downregulation of SOD was followed by the upregulation of MT (Fig. 5-15) to enhance the tolerance to thermal stress and NMs (Höckner et al. 2011, Ng et al. 2011, Gnatyshyna et al. 2012). To date no studies have been published linking the regulation of MT to increased thermal stress; however, MTs are known to contribute not only on the detoxification of metals but also to the mitigation of oxidative stress and stress in general. It is, therefore, not surprising to find a significant upregulation of this gene after thermal stress in snails exposed to CuO_Acryl_FP, which instead did not induce any mortality or any stress related genes when exposed for 10 days.

It is, in contrast, unexpected that in any of the experiments, MT was not significantly expressed when snails were exposed to Cu ions as $CuSO_4$. It is, however, possible that although the low concentrations tested were equal to ½ and ¼ of the $LC50_{96h}$ value estimated, the nominal quantity of Cu was too low to directly induce the transcription of MT. This would further corroborate the hypothesis that MT induction resulted in response to the combination of multiple stresses (NMs, long exposure and thermal stress) and not solely due to metal exposure (Gnatyshyna et al. 2012).

Exposure to $CuSO_4$, in contrast, resulted in upregulation of SOD after thermal shock, at 24 h of exposure with increasing exposure concentration; however longer exposure determined a likely exhaustion of the stress-induced antioxidant system resulting in the progressive downregulation of SOD at 96h and 10d of exposure (Fig. 5-9). Indeed, at the same time CAT was downregulated with increasing exposure concentration at 24h, indicating the inability of CAT to convert the H_2O_2 produced by SOD to O_2 and H_2O, which would reduce oxidative stress. This is reflected by the highest levels of HSP_{40},

among all the treatments, which contributed to maintain cell homeostasis mitigating the denaturation of protein, due to an excess of peroxides within the cells (Fig. 5-9).

This correlation between the antioxidant enzymes and HSPs expression was found also by Doganlar and Doganlar (2015) exposing *Drosophila melanogaster* to a mixture of pesticides for 5 days. These authors showed that increased antioxidant enzyme gene expression due to the pesticide mixture caused oxidative stress in *D. melanogaster*, which determined an increased expression in HSPs to prevent damage of proteins by ROS and correction and/or degradation of misfolded proteins.

In this research study, when snails were left 4 hours to recover from the thermal stress, independently from the form of the Cu treatment expression levels of MT remained still at the same level found after the heat shock and HSP_{40} decreased around 10-fold, remaining however, above the control threshold of 1.5-fold. This result suggested that after 4 hours of recovery from thermal stress, accumulation of aggregated oxidized proteins still follow, requiring MT to contribute to the protection to ROS and HSPs to restore the damaged proteins to their functional three-dimensional structures. Indeed, Foster et al. (2015) showed HSP_{40} expression levels induced after heat shock in the CNS of *L. stagnalis* return to control levels after 8 hours recovery from the thermal stress.

It is also worth to notice that Foster et al. (2015) studied expression levels of HSP_{40} ($\approx$ 17 fold) which were below those found in this research study ($\approx$ 40 fold) in unexposed snails. The effects observed in Foster et al (2015) may have been less severe than in the present study due to the different development stage of the snails used (adults vs juveniles). For instance, Barata et al. (2005) demonstrated an age-related decreased antioxidant enzyme activities in *D. magna* coupled with increased levels of lipid peroxidation, consistent with the oxidative stress theory of aging that implies that optimal pro/antioxidant balancing is critical for the successful aging and longevity. It is therefore likely that juvenile snails of *L. stagnalis* possess a more responsive antioxidant system than adults, given the stronger response on the HSP_{40} expression compared with the adult snails studied in Foster et al. (2015).

5.5 Conclusions

In the present study, sublethal effects of CuO NMs were studied at the molecular level, using the juvenile's stages of the pond snail *L. stagnalis*. Findings revealed that the level of stress response varies with the type of stress and if a stress is given individually or in combination with others. The combination of stresses resulted, most likely, in an enhanced pro-oxidant challenge determining, therefore, a different adaptation or

compensatory reaction toward ROS formation, to overcome the stressful condition (Regoli et al. 2011).

Generally, at the experimental conditions used, results indicate stronger significant responses produced by genes specifically involved in the stress response of the snails (HSP_{40} and MT) compared to those indicative of specific antioxidant defence, when organism are exposed to sublethal concentration of Cu. In addition, the study highlighted the increase in the induction of the defence system of the snails when subjected to heat shock, determining the significant induction of the antioxidant enzymes that otherwise were not significant transcript.

Furthermore, findings confirm the efficiency of PVP as a coating for CuO NMs to produce safer by design NMs, as demonstrated in the previous chapter using the adult stage of *L. stagnalis*. Indeed, exposure to SbyD CuO-PVP NMs resulted only in induction of the HSP_{40} after heat shock, which was attributed merely to the change in temperature rather than to the NMs.

Finally, results highlighted the need of a more holistic determination of antioxidant capacity which would provide a better understanding of an organism's resistance to toxicity caused by ROS, than the measurements of a limited number of antioxidants (Amado et al. 2009), since the antioxidant systems can act in a cooperative way as suggested by the results gathered in this research study.

Chapter 6 General Discussion

Engineered NMs are defined as two or three dimensional manufactured materials with a size range within 1-100 nm. Due to their distinctive physical-chemical proprieties, the use of these NMs, in a wide range of daily use products (*e.g.* biomedicine, bioremediation, crop protection, antimicrobial, cosmetics and electronics), has increased exponentially in the last two decades (Pu et al. 2016). However, at present, it is still not easy to quantify their release and risk to the environment and human health from nano-enabled consumer products during realistic exposure scenarios. Indeed, manufacturers often fail to provide the real quantity and composition of nano-enabled products. Furthermore, prediction of the real risk of NMs is difficult to quantify due to the wide-range of physico-chemical transformations they undergo once in the environment (soil, water and air), such as agglomeration, sorption and functionalization of other chemicals on their surface and dissolution or degradation (Baalousha et al. 2016). Different probabilistic approaches have been developed to model predicted environmental concentrations (PEC) of the different NMs, however results are usually based on local environments and difficult to extrapolate globally, due to also the poor standardization of the toxicity data produced from laboratory experiment, on which these models are based (Sun et al. 2016). Another problem is the diversity of NMs, since even NMs with the same core composition often have different sizes, morphologies and coatings.

Metal NMs, such as CuO, due to their exceptional antimicrobial proprieties, are widely used in soaps, antifouling paints and wood preservation products, posing an higher risk on the aquatic environment, in which can be introduced, for example, via directly wash-off from the treated surfaces or via BWT plants (Conway et al. 2015).

Different studies have indicated the toxicity of CuO NMs to organisms at almost all biological levels, however it is still arguable if the dissolved Cu (Adam et al. 2015, Torres-Duarte et al. 2016) or the NMs themselves (Amorim and Scott-Fordsmand 2012, Croteau et al. 2014b), or a combination of both (Mwaanga et al. 2014, Gomes et al. 2015a), are the main leads in the observed toxicity. Furthermore, most of these studies are based on the toxicity of pristine CuO NMs, which are the least likely to be found in the real environment. Indeed, as previously stated, NMs undergo different physical-chemical transformations once in the environment, which are dependent on the chemistry of the NMs and the receiving environment (Ivask et al. 2014), altering thus the toxicity that these NMs display in controlled laboratory exposure conditions (Kumar et al. 2012, Moore et al. 2016).

More, recently an increasing amount of publications have focussed on the assessment of SbyD NMs, where NMs physico-chemical proprieties (*e.g.* particles' diameter,

agglomeration behaviour, modifying surface characteristic) are controlled by their functionalization with surface coatings, in order to reduce their toxicity (Costa 2016, Hou et al. 2017a). However, in the specific case of CuO NMs, a limited amount of studies and with somehow contrasting results have been published focussing on toxicity of SbyD CuO NMs (Perreault et al. 2012, Clar et al. 2016, Javed et al. 2017, Naatz et al. 2017). Thus, more experimental research is needed to determine if the SbyD CuO NMs, once released in the environment, are able to show decreased reactivity or toxicity compared with their pristine counterpart (Nowack et al. 2012b).

This research project aimed to address this knowledge gap assessing the acute and long-term toxicity of CuO NMs at various stages of their life cycle (pristine, fragmented product and SbyD) on the benthic ecosystem, since it has been demonstrated that this compartment tends to be the environmental end-destination for particulate contaminants, including metal NMs such as CuO. The common pond snail *L. stagnalis* was chosen as a representative species of this environmental compartment, due to their wide spread worldwide distribution, the fact that they are relatively easy to rear, and reproduce, in a laboratory environment, and are considered thus good ecotoxicological models (Croteau and Luoma 2007).

Ecotoxicology experiments were performed using pristine CuO NMs, modified CuO NMs with Na ascorbate (ASC) and Polyvinylpyrrolidone (PVP) and CuO Fragmented Product (FP), where the NMs were incorporate into a matrix and then fragmented. Experiments were also carried out using soluble Cu as $CuSO_4$, to discern the contribution of Cu^{2+} on the observed toxicity effects.

Different studies have indicated the higher toxicity of NMs and heavy metals to the early stages of development of organisms compare with their adult life stage (Grosell et al. 2006, Grosell and Brix 2009, Brix et al. 2011, Brix et al. 2012, Munley et al. 2013, Niyogi et al. 2014). In this research study, no direct comparison, using the same experimental design, on the acute or chronic toxicity of CuO NMs between juveniles and adult of *L. stagnalis* was performed; however, comparable toxicity data up to 72 hrs could be extrapolated from acute juvenile and chronic adult exposures to $CuSO_4$ and pristine CuO NMs.

Results obtained confirm the higher sensitivity of juveniles to Cu in either ionic or nano form, compared with the snails' adult life stages. Indeed, $LC50_{72h}$ values estimated from the acute experiment with juveniles (Chapter 2) were, respectively, 5.97 (± 0.21 SE) and 3206.54 (± 254.61 SE) μgL^{-1} Cu for exposure to ionic Cu and pristine CuO NMs. In contrast, data extrapolated from the chronic experiments with the adult stage (≈ 22 mm)

of *L. stagnalis* (Chapter 3) revealed, at the same time point, 72 hrs, no mortality for snails exposed to $CuSO_4$ and only 20% in mortality for snails exposed to pristine CuO NMs., with an $LC20_{72h}$ value estimated of 1757.51 (± 129.8) μgL^{-1} Cu.

Furthermore, in agreement with previously published studies (Heinlaan et al. 2008, Gomes et al. 2011, Bondarenko et al. 2013, Buffet et al. 2013, Adam et al. 2015), data confirmed, in general, the higher toxicity of ionic Cu compared to the CuO NMs. Indeed, in this research study $CuSO_4$ caused higher snails' mortality, up to almost 400-fold higher, when acutely exposing (96h) juveniles of *L. stagnalis*. This great difference in lethal toxicity is markedly reduced in the chronic experiments using adult snails, where after 30 days; $CuSO_4$ was 7 times more toxic than pristine CuO NMs. A strong increase in toxicity of Cu ions compared with pristine CuO NMs was also observed when, after chronic exposure of adult snails, biological life-history traits, such as reproduction, growth (Chapter 3), and behaviour (Chapter 4) were assessed. In contrast, this marked difference was not so clear when evaluating the modulation, at molecular level, of genes (SOD, CAT, MT and HSP_{40}) indicators of the activation of a detoxification and antioxidant system. In this study, long-term experiments (10 days) were performed using juveniles, exposed to sublethal concentrations, based on the previously LC50 estimated, of either ionic Cu or CuO NMs.

Findings indicated only a general sign of stress due to the exposure to Cu ions. Indeed, exposure to Cu^{2+} as $CuSO_4$ did not significantly induce the expression of antioxidant enzymes or MT genes but only of HSP_{40}. In contrast, when snails were exposed to pristine CuO NMs, all the 4 genes selected were significantly expressed. In particular, results showed a downregulation of SOD and CAT with increasing concentrations and exposure time and an upregulation of MT followed by a high response in HSP_{40} (>3 fold). These results suggest that distinct mechanisms of toxicity are induced by Cu ions and CuO NMs. Indeed, in accordance with previous studies (Griffitt et al. 2007, Ali and Ali 2015, Siddiqui et al. 2015) findings in this project suggest a higher ROS formation due to CuO NMs compared to $CuSO_4$, indicating, thus, that CuO NMs toxicity cannot be solely attributed to dissolution.

Indeed, although contrasting dissolution rates were gathered from the characterization data (see Chapter 3, subsection 3.3.1) of the study with juveniles (exposed in 35 ml of medium) and adults (exposed in 1 L of medium), the toxicity patterns obtained were similar. In the juveniles study, the dissolution rate of CuO NMs was below 0.02% compared with the 60% found when snails were exposed in 1 L of exposure medium. Thus, if toxicity was to be attributed solely to the dissolved Cu^{2+} a correspondent

increase in toxicity should have been observed after the comparable 72h of exposure between the two experimental approaches.

It was, therefore, hypothesized, given the high agglomeration of the CuO NMs in OECD medium, that toxicity of the pristine CuO NMs was mostly due to ingestion of the NMs present at the bottom of the exposure vessel and subsequently death was caused either by impairment of the gut functionality (Croteau et al. 2011a) or internal dissolution and/or transformation of the CuO NMs in the acidic environment of digestive tract or digestive gland (Baun et al. 2008a, Golobič et al. 2012). Croteau et al. (2014a) evaluating the toxicity and uptake of Ag NMs at environmentally relevant exposures, showed that the rate constant of Ag uptake from water was faster for smaller snails compared to larger size snails, for either Ag ions or Ag NMs coated with citrate. Indeed, in this research study a higher toxicity of the same NMs was revealed in juveniles compared with adults of *L. stagnalis*.

Furthermore, even taking in consideration the difference in dissolution rate in the chronic experiment between the different CuO NMs, no proportional direct toxicity with increased dissolution was recorded in this research study. Indeed, SbyD CuO NMs functionalised in phosphate buffer presented a lower dissolution of about 30 % compared with those in Milli-Q water which was about 70% (Tab. 3-1). Nevertheless, when comparing the chronic lethal toxicity of all the SbyD CuO NMs tested at sublethal concentrations (0-200 μgL^{-1} Cu, based to the LC50 of pristine CuO NMs), findings showed an increase in toxicity due to the presence of PBS, which promoted further the agglomeration of the NMS. At that concentration range, no toxicity was recorded after exposure to pristine CuO NMs. In contrast, exposure to CuO(PO_4^{-3}) NMs induced mortality of 30% of the snails with an estimated $LC30_{30d}$ value of 186.27 (± 33.62 SE) μgL^{-1} Cu. Snails exposed to CuO-PVP(PO_4^{3-}) NMs exhibited an even higher mortality which reached almost 80% at the highest concentration tested of 200 μgL^{-1} Cu (Fig. 3-3), resulting in a LC50 value of 160.05 (± 15.65 SE) μgL^{-1} Cu. In the acute experiment, a less dramatic difference in toxicity compared to the pristine CuO NMs was observed. Results showed an acute lethal toxicity 10 times higher in juveniles exposed to CuO(PO_4^{-3}) NMs and CuO-PVP(PO_4^{-3}) compared to pristine CuO NMs (Tab. 2-1).

It is, however, important also to note that dissolution analysis was performed in abiotic conditions in the absence of organisms and food. The acute experiments were conducted in absence of food; however, it was observed, but not quantified, that snails grazed and partially excreted the CuO NMs present on the bottom of the vessels. Thus, CuO NMs would have been somehow biotransformed by the snails (Kovačec et al. 2017) changing

most likely their physico-chemical proprieties and thus toxicity. In contrast, chronic experiments were conducted in the presence of food (lettuce fed *at libitum*) which is likely to have resulted in an increase in dissolved organic matter in the exposure vessel produced by the deterioration of the uneaten lettuce leaves and the excretion of the snails for 3 days (at which time a 100% renewal of the medium was performed). Different studies have demonstrated the capability of organic matter to alter the intrinsic toxicity of NMs by surface complexation and reducing the bioavailable copper ion concentrations (Canesi et al. 2017). Furthermore, as previously demonstrated (Ng et al. 2011, Pradhan et al. 2012), Cu ions may also bind to the food left in the exposure vessel and be taken up by snails from the diet. Thus, it is clear that the mechanisms of toxicity involving all the CuO NMs tested are rather complex and cannot be solely explained by dissolution.

The hydrodynamic size and surface charge of NMs dispersions might have also affected the way in which the organisms responded to their exposures (Jiang et al. 2009). Indeed, in contrast to the previous SbyD NMs, CuO-ASC(PO_4^{-3}) induced the lowest mortality in acute exposures of juvenile snails ($LC20_{96h}$ 1471 μgL^{-1}) and in the chronic experiments (mortality below 10% at 30 days). However, this decrease in toxicity was less pronounced when evaluating life-history traits endpoints. It is possible that in the case of ASC, the amount of adsorbed coating agent on the core material, and the amount of free ASC still in suspension, might have influenced the toxicity of the two different materials, CuO-ASC(PO_4^{-3}) and CuO-ASC(H_2O) NMs. Thermographic analysis showed that in CuO-ASC(PO_4^{-3}) only 31% of the ASC was adsorbed on the core material compared to 61% for the CuO-ASC(H_2O), thus at same level of Cu concentrations the amount of free ASC in suspension was different. Ascorbate can serve either as antioxidant or pro-oxidant, depending on its quantity in dispersion (Buettner and Jurkiewicz 1996, Drouin et al. 1996). Thus, it is possible that in the case of CuO-ASC(PO_4^{-3}), given the higher quantity of free ASC in dispersion, ASC, in the short time exposure, might have acted as antioxidant potentially reducing the lethal toxicity of CuO NMs.

Findings from the sublethal endpoints evaluated during the chronic experiments of snails exposed to ionic Cu or CuO NMs indicated a direct link between the feeding rate inhibition and the decreased fecundity. This was confirmed by results gathered from the exposure to CuO-PVP(H_2O) NMs, where snails exhibited no alteration in feeding rate in response to increasing concentrations of the NMs and thus subsequently reproduction and growth were not affected in a concentration-response manner. This suggests that

PVP, in absence of PBS, was able to mitigate the toxicity of the core CuO NMs in the long-term exposure.

The same mitigating effects were shown in the behavioural study. Indeed, snails exposed to CuO-PVP(H_2O) NMs showed an alteration in the respiration behaviour only at the highest concentration tested of 200 μgL^{-1}. Snails were able to learn and form memory up to the intermediate concentration tested of 100 μgL^{-1} Cu. However, at 200 μgL^{-1} Cu, snails formed memory at the second day of training, but were not be able to retain it for more than 24 h (Fig. 4-15). Thus, as hypothesized, results confirmed the highest sensitivity of LTM test demonstrated in the detection of early stress symptoms due to exposure to NMs compared to more conventional endpoints, such as LCx. Indeed, the inability of snails to form memory can be attributed to a damage in their CNS, due to exposure to the NMs. Furthermore, reproduction in *L. stagnalis* is controlled by neurons involved in the egg laying and sexual behaviour regulation, such gonadotropin-releasing hormone (GnRH) peptides (Young et al. 1999).Therefore, it is not unexpected that EC_{30d} of either ionic Cu or CuO NMs from the behaviour studies were, overall, lower than the EC_{30d} values determined for the reproduction study, indicating an early toxic effect of Cu on the normal neurophysiological function of the snails.

Finally, results from the long-term exposure tests of juveniles of *Lymnaea* at sublethal concentrations of Cu can be correlated with the LTM test findings. Indeed, Sunada et al. (2016) demonstrated that thermal stress enhanced the formation of memory on *L. stagnalis* due to the activation of HSPs. This was further confirmed by experiments performed after snails were injected with quercetin or 5-AZA, which are respectively blockers for HSPs activation and DNA methylation. In this case, snails did not exhibit any memory enhancement. However, the authors drew their conclusion based solely on the alteration of the snails' behaviour, no HSPs activity nor gene expression induction were quantified (Sunada et al. 2016).

In this research study, a direct relationship between memory formation and HSPs cannot be made, given that gene expression analyses were conducted, using juveniles and the behaviour study used adults of *L. stagnalis*. Nevertheless, as demonstrated by previous authors (Boon-Niermeijer et al. 1986, Foster et al. 2015, Sunada et al. 2016, Kim et al. 2018), when a thermal stress was applied independently from the chemical, a marked upregulation of HSP_{40} was observed, suggesting that similar thermoregulation mechanisms are activated at different life stages.

Indeed, for example, exposure to CuO-PVP(H_2O) NMs, which was shown to be relatively non-toxic to *Lymnaea*, resulted only in the induction of the HSP_{40} after heat shock, which was attributed merely to the change in temperature rather than to the exposure to the NMs.

This research project developed a holistic approach to the evaluation of the toxicity of different forms of CuO NMs, via acute and chronic exposure using different life stages of *L. stagnalis* and biological endpoints (mortality, reproduction and behaviour studies and molecular response) under environment stressors. This project has determined that effects of exposure to Cu, under different forms, ionic and nano, cannot always be attributed to dissolved copper.

Furthermore, findings highlighted the importance of considering the exposure conditions during to formulate efficient SbyD CuO NMs.

Further studies, however, should be conducted to discern the mechanism of toxicity of the different materials, with attention to the action of the different coatings and dispersants in determining fate and behaviour of CuO NMs in realistic environmental exposure conditions and leading to specific effects.

References

Adam, N., Vakurov, A., Knapen, D. and Blust, R. (2015) 'The chronic toxicity of CuO nanoparticles and copper salt to Daphnia magna', *Journal of Hazardous Materials*, 283, 416-422.

Adeleye, A. S., Conway, J. R., Perez, T., Rutten, P. and Keller, A. A. (2014) 'Influence of extracellular polymeric substances on the long-term fate, dissolution, and speciation of copper-based nanoparticles', *Environmental Science & Technology*, 48(21), 12561-12568.

Adeleye, A. S., Oranu, E. A., Tao, M. Y. and Keller, A. A. (2016) 'Release and detection of nanosized copper from a commercial antifouling paint', *Water Research*, 102, 374-382.

Al-Bairuty, G. A., Shaw, B. J., Handy, R. D. and Henry, T. B. (2013) 'Histopathological effects of waterborne copper nanoparticles and copper sulphate on the organs of rainbow trout (*Oncorhynchus mykiss*)', *Aquatic Toxicology*, 126, 104-115.

Al-Shaeri, M., Ahmed, D., McCluskey, F., Turner, G., Paterson, L., Dyrynda, E. A. and Hartl, M. G. J. (2013) 'Potentiating toxicological interaction of single-walled carbon nanotubes with dissolved metals', *Environmental Toxicology and Chemistry*, 32(12), 2701-2710.

Ali, D., Alarifi, S., Kumar, S., Ahamed, M. and Siddiqui, M. A. (2012) 'Oxidative stress and genotoxic effect of zinc oxide nanoparticles in freshwater snail *Lymnaea luteola L*', *Aquatic Toxicology*, 124-125, 83-90.

Ali, D. and Ali, H. (2015) 'Susceptibility of the freshwater pulmonate snail *Lymnea luteola L.* to copper oxide nanoparticle', *Toxicological & Environmental Chemistry*, 97(5), 576-587.

Amado, L. L., Garcia, M. L., Ramos, P. B., Freitas, R. F., Zafalon, B., Ferreira, J. L. R., Yunes, J. S. and Monserrat, J. M. (2009) 'A method to measure total antioxidant capacity against peroxyl radicals in aquatic organisms: Application to evaluate microcystins toxicity', *Science of The Total Environment*, 407(6), 2115-2123.

Ambali, S. F., Idris, S. B., Onukak, C., Shittu, M. and Ayo, J. O. (2010) 'Ameliorative effects of vitamin C on short-term sensorimotor and cognitive changes induced by acute chlorpyrifos exposure in Wistar rats', *Toxicology and Industrial Health*, 26(9), 547-558.

Amde, M., Liu, J.-f., Tan, Z.-Q. and Bekana, D. (2017) 'Transformation and bioavailability of metal oxide nanoparticles in aquatic and terrestrial environments. A review', *Environmental Pollution*, 230, 250-267.

Amorim, M. J. B. and Scott-Fordsmand, J. J. (2012) 'Toxicity of copper nanoparticles and $CuCl_2$ salt to *Enchytraeus albidus* worms: Survival, reproduction and avoidance responses', *Environmental Pollution*, 164, 164-168.

An, L., Liu, S., Yang, Z. and Zhang, T. (2012) 'Cognitive impairment in rats induced by nano-CuO and its possible mechanisms', *Toxicology Letters*, 213(2), 220-227.

Andrade, V. M., Aschner, M. and Marreilha dos Santos, A. P. (2017) 'Neurotoxicity of metal mixtures' in Aschner, M. and Costa, L. G., eds., *Neurotoxicity of Metals*, Cham: Springer International Publishing, 227-265.

Aono, K., Fusada, A., Fusada, Y., Ishii, W., Kanaya, Y., Komuro, M., Matsui, K., Meguro, S., Miyamae, A. and Miyamae, Y. (2008) 'Upside-down gliding of *Lymnaea*', *The Biological Bulletin*, 215(3), 272-279.

Apte, S. C., Batley, G. E., Bowles, K. C., Brown, P. L., Creighton, N., Hales, L. T., Hyne, R. V., Julli, M., Markich, S. J. and Pablo, F. (2006) 'A comparison of copper speciation measurements with the toxic responses of three sensitive freshwater organisms', *Environmental Chemistry*, 2(4), 320-330.

Arambaši, M. B., Paši, M., Ristanovi, D., Kalauzi, A. and Koji, L. (2013) 'Pond snail *Lymnaea stagnalis L.*: The implication for basic and applied research', *World Applied Sciences Journal*, 25(10), 1438-1448.

Arambaši, M. B., PAšć, M., Koć, L., Kalauzi, A. and MARKović, V. (1987) 'The growth of pond snali *Lymnaea stagnalis L.* in laboratory conditions', *Zoologische Jahrbücher. Abteilung für Anatomie und Ontogenie der Tier*, 16, 119-128.

Ates, M., Arslan, Z., Demir, V., Daniels, J. and Farah, I. O. (2015) 'Accumulation and toxicity of CuO and ZnO nanoparticles through waterborne and dietary exposure of goldfish (*Carassius auratus*)', *Environmental Toxicology*, 30(1), 119-128.

Atli, G. and Grosell, M. (2016) 'Characterization and response of antioxidant systems in the tissues of the freshwater pond snail *(Lymnaea stagnalis*) during acute copper exposure', *Aquatic Toxicology*, 176, 38-44.

Axenov-Gribanov, D., Vereshchagina, K., Lubyaga, Y., Gurkov, A., Bedulina, D., Shatilina, Z., Khomich, A., Golubev, A. and Timofeyev, M. (2015) 'Stress response at the cellular and biochemical levels indicates the limitation of the environmental temperature range for eastern Siberian populations of the common gastropod *Lymnaea stagnalis*', *Malacologia*, 59(1), 33-44.

Azami, S., Wagatsuma, A., Sadamoto, H., Hatakeyama, D., Usami, T., Fujie, M., Koyanagi, R., Azumi, K., Fujito, Y., Lukowiak, K. and Ito, E. (2006) 'Altered gene activity correlated with long-term memory formation of conditioned taste aversion in *Lymnaea*', *Journal of Neuroscience Research*, 84(7), 1610-1620.

Baalousha, M., Cornelis, G., Kuhlbusch, T. A. J., Lynch, I., Nickel, C., Peijnenburg, W. and van den Brink, N. W. (2016) 'Modeling nanomaterial fate and uptake in the environment: current knowledge and future trends', *Environmental Science: Nano*, 3(2), 323-345.

Baalousha, M., Ju-Nam, Y., Cole, P. A., Gaiser, B., Fernandes, T. F., Hriljac, J. A., Jepson, M. A., Stone, V., Tyler, C. R. and Lead, J. R. (2012) 'Characterization of cerium oxide nanoparticles—Part 1: Size measurements', *Environmental Toxicology and Chemistry*, 31(5), 983-993.

Barata, C., Baird, D. J., Nogueira, A. J. A., Soares, A. M. V. M. and Riva, M. C. (2006) 'Toxicity of binary mixtures of metals and pyrethroid insecticides to *Daphnia magna Straus*. Implications for multi-substance risks assessment', *Aquatic Toxicology*, 78(1), 1-14.

Barata, C., Carlos Navarro, J., Varo, I., Carmen Riva, M., Arun, S. and Porte, C. (2005) 'Changes in antioxidant enzyme activities, fatty acid composition and lipid peroxidation in *Daphnia magna* during the aging process', *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 140(1), 81-90.

Baumann, J., Köser, J., Arndt, D. and Filser, J. (2014) 'The coating makes the difference: Acute effects of iron oxide nanoparticles on *Daphnia magna*', *Science of The Total Environment*, 484(Supplement C), 176-184.

Baun, A., Hartmann, N. B., Grieger, K. and Kusk, K. O. (2008a) 'Ecotoxicity of engineered nanoparticles to aquatic invertebrates: a brief review and recommendations for future toxicity testing', *Ecotoxicology*, 17(5), 387-395.

Baun, A., Sørensen, S. N., Rasmussen, R. F., Hartmann, N. B. and Koch, C. B. (2008b) 'Toxicity and bioaccumulation of xenobiotic organic compounds in the presence of aqueous suspensions of aggregates of nano-C_{60}', *Aquatic Toxicology*, 86(3), 379-387.

Besser, J. M., Dorman, R. A., Hardesty, D. L. and Ingersoll, C. G. (2016) 'Survival and growth of freshwater pulmonate and nonpulmonate snails in 28-day exposures to copper, ammonia, and pentachlorophenol', *Archives of Environmental Contamination and Toxicology*, 70(2), 321-331.

Bhatia, S. (2016) 'Nanoparticles types, classification, characterization, fabrication methods and drug delivery applications' in *Natural Polymer Drug Delivery Systems: Nanoparticles, Plants, and Algae*, Cham: Springer International Publishing, 33-93.

Bicho, R. C., Santos, F. C. F., Scott-Fordsmand, J. J. and Amorim, M. J. B. (2017) 'Effects of copper oxide nanomaterials (CuO NMs) are life stage dependent – full life cycle in *Enchytraeus crypticus*', *Environmental Pollution*, 224(Supplement C), 117-124.

Bleeker, E. A. J., de Jong, W. H., Geertsma, R. E., Groenewold, M., Heugens, E. H. W., Koers-Jacquemijns, M., van de Meent, D., Popma, J. R., Rietveld, A. G., Wijnhoven, S. W. P., Cassee, F. R. and Oomen, A. G. (2013) 'Considerations on the EU definition of a nanomaterial: Science to support policy making', *Regulatory Toxicology and Pharmacology*, 65(1), 119-125.

Blinova, I., Ivask, A., Heinlaan, M., Mortimer, M. and Kahru, A. (2010) 'Ecotoxicity of nanoparticles of CuO and ZnO in natural water', *Environmental Pollution*, 158(1), 41-47.

Bondarenko, O., Juganson, K., Ivask, A., Kasemets, K., Mortimer, M. and Kahru, A. (2013) 'Toxicity of Ag, CuO and ZnO nanoparticles to selected environmentally relevant test organisms and mammalian cells in vitro: a critical review', *Archives of Toxicology*, 87(7), 1181-1200.

Boon-Niermeijer, E. K., Tuyl, M. and van de Scheur, H. (1986) 'Evidence for two states of thermotolerance', *International Journal of Hyperthermia*, 2(1), 93-105.

Boss, C. and J. Fredeen, K. (2004) *Concepts, instrumentation and techniques in inductively coupled plasma optical emission spectrometry*, Third Edition ed., PerkinElmer, Inc.

Bottero, J. Y., Rose, J., de Garidel, C., Masion, A., Deutsch, T., Brochard, G., Carriere, M., Gontard, N., Wortham, H., Rabilloud, T., Salles, B., Dubosson, M., Cathala, B., Boutry, D., Ereskovsky, A., Auplat, C., Charlet, L., Heulin, T., Frejafon, E. and Lanone, S. (2017) 'SERENADE: safer and ecodesign research and education applied to nanomaterial development, the new generation of materials safer by design', *Environmental Science-Nano*, 4(3), 526-538.

Bouwmeester, H., Lynch, I., marvin, H. J. P., Dawson, K. A., Berges, M., Braguer, D., Byrne, H. J., Casey, A., Chambers, G., Clift, M. J. D., Elia, G., Fernandes, T. F., Fjellsbø, L. B., Hatto, P., Juillerat, L., Klein, C., Kreyling, W. G., Nickel, C., Riediker, M. and Stone, V. (2011) 'Minimal analytical characterization of engineered nanomaterials needed for hazard assessment in biological matrices', *Nanotoxicology*, 5(1), 1-11.

Brix, K. V., Esbaugh, A. J. and Grosell, M. (2011) 'The toxicity and physiological effects of copper on the freshwater pulmonate snail, *Lymnaea stagnalis*', *Comparative Biochemistry and Physiology - Part C: Toxicology & Pharmacology*, 154(3), 261-7.

Brix, K. V., Esbaugh, A. J., Munley, K. M. and Grosell, M. (2012) 'Investigations into the mechanism of lead toxicity to the freshwater pulmonate snail, *Lymnaea stagnalis*', *Aquatic Toxicology*, 106–107, 147-156.

Brunelli, A., Zabeo, A., Semenzin, E., Hristozov, D. and Marcomini, A. (2016) 'Extrapolated long-term stability of titanium dioxide nanoparticles and multi-walled carbon nanotubes in artificial freshwater', *Journal of Nanoparticle Research*, 18(5), 113.

Buettner, G. R. and Jurkiewicz, B. A. (1996) 'Catalytic metals, ascorbate and free radicals: combinations to avoid', *Radiation research*, 145(5), 532-541.

Buffet, P.-E., Richard, M., Caupos, F., Vergnoux, A., Perrein-Ettajani, H., Luna-Acosta, A., Akcha, F., Amiard, J.-C., Amiard-Triquet, C. and Guibbolini, M. (2013) 'A mesocosm study of fate and effects of CuO nanoparticles on endobenthic species (*Scrobicularia plana*, *Hediste diversicolor*)', *Environmental Science & Technology*, 47(3), 1620-1628.

Buffet, P.-E., Tankoua, O. F., Pan, J.-F., Berhanu, D., Herrenknecht, C., Poirier, L., Amiard-Triquet, C., Amiard, J.-C., Bérard, J.-B. and Risso, C. (2011) 'Behavioural and biochemical responses of two marine invertebrates *Scrobicularia plana* and *Hediste diversicolor* to copper oxide nanoparticles', *Chemosphere*, 84(1), 166-174.

Bulcke, F., Dringen, R. and Scheiber, I. F. (2017) 'Neurotoxicity of Copper' in Aschner, M. and Costa, L. G., eds., *Neurotoxicity of Metals*, Cham: Springer International Publishing, 313-343.

Bundschuh, M., Seitz, F., Rosenfeldt, R. R. and Schulz, R. (2016) 'Effects of nanoparticles in fresh waters: risks, mechanisms and interactions', *Freshwater Biology*, 61(12), 2185-2196.

Byzitter, J., Lukowiak, K., Karnik, V. and Dalesman, S. (2012) 'Acute combined exposure to heavy metals (Zn, Cd) blocks memory formation in a freshwater snail', *Ecotoxicology*, 21(3), 860-868.

Caballero-Guzman, A. and Nowack, B. (2016) 'A critical review of engineered nanomaterial release data: Are current data useful for material flow modeling?', *Environmental Pollution*, 213, 502-517.

Caballero-Guzman, A. and Nowack, B. (2017) *Environmental concentrations of nanomaterials released from four applications during their life cycle: do they influence the entire system?*, unpublished.

Canesi, L., Balbi, T., Fabbri, R., Salis, A., Damonte, G., Volland, M. and Blasco, J. (2017) 'Biomolecular coronas in invertebrate species: Implications in the environmental impact of nanoparticles', *NanoImpact*, 8, 89-98.

Charles, S., Ducrot, V., Azam, D., Benstead, R., Brettschneider, D., De Schamphelaere, K., Filipe Goncalves, S., Green, J. W., Holbech, H., Hutchinson, T. H., Faber, D., Laranjeiro, F., Matthiessen, P., Norrgren, L., Oehlmann, J., Reategui-Zirena, E., Seeland-Fremer, A., Teigeler, M., Thome, J.-P., Tobor Kaplon, M., Weltje, L. and Lagadic, L. (2016) 'Optimizing the design of a reproduction toxicity test with the pond snail *Lymnaea stagnalis*', *Regulatory Toxicology and Pharmacology*, 81, 47-56.

Clar, J. G., Li, X., Impellitteri, C. A., Bennett-Stamper, C. and Luxton, T. P. (2016) 'Copper nanoparticle induced cytotoxicity to nitrifying bacteria in wastewater treatment: A mechanistic copper speciation study by x-ray absorption spectroscopy', *Environmental Science & Technology*, 50(17), 9105-9113.

Clogston, J. D. and Patri, A. K. (2011) 'Zeta potential measurement' in McNeil, S. E., ed. *Characterization of Nanoparticles Intended for Drug Delivery*, Totowa, NJ: Humana Press, 63-70.

Coggins, B. L., Collins, J. W., Holbrook, K. J. and Yampolsky, L. Y. (2017) 'Antioxidant capacity, lipid peroxidation, and lipid composition changes during long-term and short-term thermal acclimation in *Daphnia*', *Journal of Comparative Physiology B*, 187(8), 1091-1106.

Conway, J. R., Adeleye, A. S., Gardea-Torresdey, J. and Keller, A. A. (2015) 'Aggregation, dissolution, and transformation of copper nanoparticles in natural waters', *Environmental Science & Technology*, 49(5), 2749-2756.

Costa, A. L. (2016) 'Applying safety by molecular design concepts to nanomaterials risk management' in *Managing Risk in Nanotechnology: Topics in Governance, Assurance and Transfer*, Cham: Springer International Publishing, 171-195.

Covich, A. P., Palmer, M. A. and Crowl, T. A. (1999) 'The role of benthic invertebrate species in freshwater ecosystems: Zoobenthic species influence energy flows and nutrient cycling', *BioScience*, 49(2), 119-127.

Croteau, M.-N., Dybowska, A. D., Luoma, S. N. and Valsami-Jones, E. (2011a) 'A novel approach reveals that zinc oxide nanoparticles are bioavailable and toxic after dietary exposures', *Nanotoxicology*, 5(1), 79-90.

Croteau, M.-N., Misra, S. K., Luoma, S. N. and Valsami-Jones, E. (2011b) 'Silver bioaccumulation dynamics in a freshwater invertebrate after aqueous and dietary exposures to nanosized and ionic Ag', *Environmental Science & Technology*, 45(15), 6600-6607.

Croteau, M. N., Dybowska, A. D., Luoma, S. N., Misra, S. K. and Valsami-Jones, E. (2014a) 'Isotopically modified silver nanoparticles to assess nanosilver bioavailability and toxicity at environmentally relevant exposures', *Environmental Chemistry*, 11(3), 247-256.

Croteau, M. N. and Luoma, S. N. (2007) 'Characterizing dissolved Cu and Cd uptake in terms of the biotic ligand and biodynamics using enriched stable isotopes', *Environmental Science & Technology*, 41(9), 3140-5.

Croteau, M. N., Misra, S. K., Luoma, S. N. and Valsami-Jones, E. (2014b) 'Bioaccumulation and toxicity of CuO nanoparticles by a freshwater invertebrate after waterborne and dietborne exposures', *Environmental Science & Technology*, 48(18), 10929-10937.

Côte, J., Bouétard, A., Pronost, Y., Besnard, A.-L., Coke, M., Piquet, F., Caquet, T. and Coutellec, M.-A. (2015) 'Genetic variation of *Lymnaea stagnalis* tolerance to copper: A test of selection hypotheses and its relevance for ecological risk assessment', *Environmental Pollution*, 205(Supplement C), 209-217.

Dalesman, S. and Rundle, S. D. (2010) 'Influence of rearing and experimental temperatures on predator avoidance behaviour in a freshwater pulmonate snail', *Freshwater Biology*, 55(10), 2107-2113.

Dalesman, S., Rundle, S. D. and Lukowiak, K. (2011) 'Microgeographical variability in long-term memory formation in the pond snail, *Lymnaea stagnalis*', *Animal Behaviour*, 82(2), 311-319.

Dallinger, R., Chabicovsky, M., Hödl, E., Prem, C., Hunziker, P. and Manzl, C. (2005) 'Copper in *Helix pomatia* (Gastropoda) is regulated by one single cell type: differently responsive metal pools in rhogocytes', *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 289(4), R1185-R1195.

Das, S. and Khangarot, B. S. (2011) 'Bioaccumulation of copper and toxic effects on feeding, growth, fecundity and development of pond snail *Lymnaea luteola* L', *Journal of Hazardous Materials*, 185(1), 295-305.

Dawson, A., Baxendale, D. A. and Wood, E. J. (1981) 'Equilibrium and kinetic studies of oxygen binding by gastropod (*Lymnaea stagnalis*) haemocyanin', *Biochemical Society Transactions,* 9(5), 446-447.

De Schamphelaere, K. A. C., Koene, J. M., Heijerick, D. G. and Janssen, C. R. (2008) 'Reduction of growth and haemolymph Ca levels in the freshwater snail *Lymnaea stagnalis* chronically exposed to cobalt', *Ecotoxicology and Environmental Safety,* 71(1), 65-70.

De Visser, J. A. G. M., Maat, A. T. and Zonneveld, C. (1994) 'Energy budgets and reproductive allocation in the simultaneous hermaphrodite pond snail, *Lymnaea stagnalis* (*L.*): A trade-off between male and female function', *The American Naturalist,* 144(5), 861-867.

den Besten, P. J. and Munawar, M. (2005) *Ecotoxicological testing of marine and freshwater ecosystems: Emerging techniques, trends and strategies,* 1st edition ed., Boca Raton: CRC Press.

Desouky, M. M. A. (2006) 'Tissue distribution and subcellular localization of trace metals in the pond snail *Lymnaea stagnalis* with special reference to the role of lysosomal granules in metal sequestration', *Aquatic Toxicology,* 77(2), 143-152.

Dhawan, A., Sharma, V. and Parmar, D. (2009) 'Nanomaterials: a challenge for toxicologists', *Nanotoxicology,* 3(1), 1-9.

Doganlar, O. and Doganlar, B. Z. (2015) 'Responses of antioxidant enzymes and heat shock proteins in *Drosophila* to treatment with a pesticide mixture', *Archives of Biological Sciences,* 67(3), 869-876.

Drouin, R., Rodriguez, H., Gao, S.-W., Gebreyes, Z., O'Connor, T. R., Holmquist, G. P. and Akman, S. A. (1996) 'Cupric ion/ascorbate/hydrogen peroxide-induced DNA damage: DNA-bound copper ion primarily induces base modifications', *Free Radical Biology and Medicine,* 21(3), 261-273.

Ducrot, V., Askem, C., Azam, D., Brettschneider, D., Brown, R., Charles, S., Coke, M., Collinet, M., Delignette-Muller, M. L., Forfait-Dubuc, C., Holbech, H., Hutchinson, T., Jach, A., Kinnberg, K. L., Lacoste, C., Le Page, G., Matthiessen, P., Oehlmann, J., Rice, L., Roberts, E., Ruppert, K., Davis, J. E., Veauvy, C., Weltje, L., Wortham, R. and Lagadic, L. (2014) 'Development and validation of an OECD reproductive toxicity test guideline with the pond snail *Lymnaea stagnalis* (Mollusca, Gastropoda)', *Regulatory Toxicology and Pharmacology,* 70(3), 605-614.

Dummee, V., Tanhan, P., Kruatrachue, M., Damrongphol, P. and Pokethitiyook, P. (2015) 'Histopathological changes in snail, *Pomacea canaliculata*, exposed to sub-lethal copper sulfate concentrations', *Ecotoxicology and Environmental Safety*, 122(Supplement C), 290-295.

Ebanks, S. C. and Grosell, M. (2008) 'Fluid and osmolyte recovery in the common pond snail *Lymnaea stagnalis* following full-body withdrawal', *Journal of Experimental Biology*, 211(3), 327.

EU, C. (1993) 'Commission Directive 93/67/EEG of 20 July 1993, laying down the principles for the assessment of risks to man and the environment of substances notified in accordance with Council Directive 67/548/EEG'.

EU, C. (2011) 'Commission Recommendation of 18 October 2011 on the definition of nanomaterial (2011/696/EU)', *Official Journal of the European Communities: Legis*.

Fan, C.-Y., Lee, S. and Cyr, D. M. (2003) 'Mechanisms for regulation of Hsp70 function by Hsp40', *Cell Stress & Chaperones*, 8(4), 309-316.

Fei, G. H. and Feng, Z. P. (2008) 'Chronic hypoxia-induced alteration of presynaptic protein profiles and neurobehavioral dysfunction are averted by supplemental oxygen in *Lymnaea stagnalis*', *Neuroscience*, 153(1), 318-328.

Felix, L. C., Ortega, V. A. and Goss, G. G. (2017) 'Cellular uptake and intracellular localization of poly (acrylic acid) nanoparticles in a rainbow trout (*Oncorhynchus mykiss*) gill epithelial cell line, RTgill-W1', *Aquatic Toxicology*, 192, 58-68.

Fent, K. (2010) 'Ecotoxicology of Engineered Nanoparticles' in Frimmel, F. H. and Niessner, R., eds., *Nanoparticles in the Water Cycle: Properties, Analysis and Environmental Relevance*, Berlin, Heidelberg: Springer Berlin Heidelberg, 183-205.

Foster, N. L., Lukowiak, K. and Henry, T. B. (2015) 'Time-related expression profiles for heat shock protein gene transcripts (HSP40, HSP70) in the central nervous system of *Lymnaea stagnalis* exposed to thermal stress', *Communicative & Integrative Biology*, 8(3), e1040954.

Fukui, H., Iwahashi, H., Nishio, K., Hagihara, Y., Yoshida, Y. and Horie, M. (2017) 'Ascorbic acid prevents zinc oxide nanoparticle–induced intracellular oxidative stress and inflammatory responses', *Toxicology and Industrial Health*, 33(9), 687-695.

Gao, L., Doan, H., Nidumolu, B., Kumar, A. and Gonzago, D. (2017) 'Effects of copper on the survival, hatching, and reproduction of a pulmonate snail (*Physa acuta*)', *Chemosphere,* 185(Supplement C), 1208-1216.

Garner, K. L. and Keller, A. A. (2014) 'Emerging patterns for engineered nanomaterials in the environment: a review of fate and toxicity studies', *Journal of Nanoparticle Research,* 16(8), 2503.

Gleason, L. U. and Burton, R. S. (2015) 'RNA-seq reveals regional differences in transcriptome response to heat stress in the marine snail *Chlorostoma funebralis*', *Molecular ecology,* 24(3), 610-627.

Gnatyshyna, L., Falfushynska, H., Bodilovska, O., Oleynik, O., Golubev, A. and Stoliar, O. (2012) 'Metallothionein and glutathione in *Lymnaea stagnalis* determine the specificity of responses to the effects of ionising radiation', *Radioprotection,* 47(2), 231-242.

Goldstein, S. and Czapski, G. (1986) 'The role and mechanism of metal ions and their complexes in enhancing damage in biological systems or in protecting these systems from these systems from the toxicity of O_2^-', *Journal of Free Radicals in Biology & Medicine,* 2(1), 3-11.

Golobič, M., Jemec, A., Drobne, D., Romih, T., Kasemets, K. and Kahru, A. (2012) 'Upon exposure to Cu nanoparticles, accumulation of cpper in the iopod *Porcellio scaber* is due to the dissolved Cu ions inside the digestive tract', *Environmental Science & Technology,* 46(21), 12112-12119.

Gomes, S. I., Hansen, D., Scott-Fordsmand, J. J. and Amorim, M. J. (2015a) 'Effects of silver nanoparticles to soil invertebrates: oxidative stress biomarkers in *Eisenia fetida*', *Environmental pollution,* 199, 49-55.

Gomes, S. I., Murphy, M., Nielsen, M. T., Kristiansen, S. M., Amorim, M. J. B. and Scott-Fordsmand, J. J. (2015b) 'Cu-nanoparticles ecotoxicity – Explored and explained?', *Chemosphere,* 139, 240-245.

Gomes, T., Pereira, C. G., Cardoso, C., Pinheiro, J. P., Cancio, I. and Bebianno, M. J. (2012) 'Accumulation and toxicity of copper oxide nanoparticles in the digestive gland of *Mytilus galloprovincialis*', *Aquatic Toxicology,* 118-119(Supplement C), 72-79.

Gomes, T., Pinheiro, J. P., Cancio, I., Pereira, C. G., Cardoso, C. and Bebianno, M. J. (2011) 'Effects of copper nanoparticles exposure in the mussel *Mytilus galloprovincialis*', *Environmental Science & Technology,* 45(21), 9356-9362.

Gottschalk, F., Sun, T. Y. and Nowack, B. (2013) 'Environmental concentrations of engineered nanomaterials: Review of modeling and analytical studies', *Environmental Pollution*, 181, 287-300.

Griffith, M. B. (2017) 'Toxicological perspective on the osmoregulation and ionoregulation physiology of major ions by freshwater animals: Teleost fish, crustacea, aquatic insects, and Mollusca', *Environmental Toxicology and Chemistry*, 36(3), 576-600.

Griffitt, R. J., Luo, J., Gao, J., Bonzongo, J.-C. and Barber, D. S. (2008) 'Effects of particle composition and species on toxicity of metallic nanomaterials in aquatic organisms', *Environmental Toxicology and Chemistry*, 27(9), 1972-1978.

Griffitt, R. J., Weil, R., Hyndman, K. A., Denslow, N. D., Powers, K., Taylor, D. and Barber, D. S. (2007) 'Exposure to copper nanoparticles causes gill injury and acute lethality in zebrafish (*Danio rerio*)', *Environmental Science & Technology*, 41(23), 8178-8186.

Grosell, M. and Brix, K. V. (2009) 'High net calcium uptake explains the hypersensitivity of the freshwater pulmonate snail, *Lymnaea stagnalis*, to chronic lead exposure', *Aquatic Toxicology*, 91(4), 302-311.

Grosell, M., Gerdes, R. M. and Brix, K. V. (2006) 'Chronic toxicity of lead to three freshwater invertebrates - *Brachionus calyciflorus*, *Chironomus tentans*, and *Lymnaea stagnalis*', *Environmental Toxicology and Chemistry*, 25(1), 97-104.

Grosell, M., Nielsen, C. and Bianchini, A. (2002) 'Sodium turnover rate determines sensitivity to acute copper and silver exposure in freshwater animals', *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 133(1), 287-303.

Gunawan, C., Teoh, W. Y., Marquis, C. P. and Amal, R. (2011) 'Cytotoxic origin of copper(II) oxide nanoparticles: Comparative studies with micron-sized particles, leachate, and metal salts', *ACS Nano*, 5(9), 7214-7225.

Handy, R. D., von der Kammer, F., Lead, J. R., Hassellov, M., Owen, R. and Crane, M. (2008) 'The ecotoxicology and chemistry of manufactured nanoparticles', *Ecotoxicology*, 17(4), 287-314.

Hanna, S., Miller, R. and Lenihan, H. (2014) 'Accumulation and toxicity of copper oxide engineered nanoparticles in a marine mussel', *Nanomaterials*, 4(3), 535.

Hanna, S. K., Miller, R. J., Zhou, D., Keller, A. A. and Lenihan, H. S. (2013) 'Accumulation and toxicity of metal oxide nanoparticles in a soft-sediment estuarine amphipod', *Aquatic Toxicology*, 142–143, 441-446.

Heinlaan, M., Ivask, A., Blinova, I., Dubourguier, H.-C. and Kahru, A. (2008) 'Toxicity of nanosized and bulk ZnO, CuO and TiO_2 to bacteria *Vibrio fischeri* and crustaceans *Daphnia magna* and *Thamnocephalus platyurus*', *Chemosphere*, 71(7), 1308-1316.

Heinlaan, M., Kahru, A., Kasemets, K., Arbeille, B., Prensier, G. and Dubourguier, H.-C. (2011) 'Changes in the *Daphnia magna* midgut upon ingestion of copper oxide nanoparticles: A transmission electron microscopy study', *Water Research*, 45(1), 179-190.

Henry, T. B., Wileman, S. J., Boran, H. and Sutton, P. (2013) 'Association of Hg^{2+} with Aqueous $(C\text{-}60)_n$ Aggregates Facilitates Increased Bioavailability of Hg^{2+} in Zebrafish (*Danio rerio*)', *Environmental Science & Technology*, 47(17), 9997-10004.

Hochella Jr, M., Aruguete, D., Kim, B. and Madden, A. (2012) 'Naturally occurring inorganic nanoparticles: General assessment and a global budget for one of Earth's last unexplored geochemical components', *Nature's Nanostructures*, 1-42.

Hoefnagel, K. N. and Verberk, W. C. E. P. (2017) 'Long-term and acute effects of temperature and oxygen on metabolism, food intake, growth and heat tolerance in a freshwater gastropod', *Journal of Thermal Biology*, 68, 27-38.

Homa, J., Stürzenbaum, S. R. and Kolaczkowska, E. (2016) 'Metallothionein 2 and heat shock protein 72 protect *Allolobophora chlorotica* from cadmium but not nickel or copper exposure: Body malformation and coelomocyte functioning', *Archives of Environmental Contamination and Toxicology*, 71(2), 267-277.

Hou, J., Wang, X., Hayat, T. and Wang, X. (2017a) 'Ecotoxicological effects and mechanism of CuO nanoparticles to individual organisms', *Environmental Pollution*, 221(Supplement C), 209-217.

Hou, J., Zhou, Y., Wang, C., Li, S. and Wang, X. (2017b) 'Toxic effects and molecular mechanism of different types of silver nanoparticles to the aquatic crustacean *Daphnia magna*', *Environmental Science & Technology*, 51(21), 12868-12878.

Hughes, E., Shymansky, T., Swinton, E., Lukowiak, K. S., Swinton, C., Sunada, H., Protheroe, A., Phillips, I. and Lukowiak, K. (2017) 'Strain-specific differences of the effects of stress on memory in *Lymnaea*', *The Journal of Experimental Biology*, 220(5), 891-899.

Hughes, M. N. and Poole, R. K. (1991) 'Metal speciation and microbial growth—the hard (and soft) facts', *Microbiology*, 137(4), 725-734.

Hwang, Y., Lee, J.-K., Lee, J.-K., Jeong, Y.-M., Cheong, S.-i., Ahn, Y.-C. and Kim, S. H. (2008) 'Production and dispersion stability of nanoparticles in nanofluids', *Powder Technology*, 186(2), 145-153.

Höckner, M., Dallinger, R. and Stürzenbaum, S. R. (2011) 'Nematode and snail metallothioneins', *JBIC Journal of Biological Inorganic Chemistry*, 16(7), 1057.

Inoue, T., Takasaki, M., Lukowiak, K. and Syed, N. (1996) 'Inhibition of the respiratory pattern-generating neurons by an identified whole-body withdrawal interneuron of Lymnaea stagnalis', *The Journal of Experimental Biology*, 199(9), 1887-1898.

ISO (2015) 'ISO/TS 80004-2:2015 Nanotechnologies - Vocabulary - Part 2: Nano-objects', 10.

Ivask, A., Juganson, K., Bondarenko, O., Mortimer, M., Aruoja, V., Kasemets, K., Blinova, I., Heinlaan, M., Slaveykova, V. and Kahru, A. (2014) 'Mechanisms of toxic action of Ag, ZnO and CuO nanoparticles to selected ecotoxicological test organisms and mammalian cells in vitro: A comparative review', *Nanotoxicology*, 8(sup1), 57-71.

Jacobsen, N., Pojano, G., Wallin, H. and Jensen, K. (2010) *Nanomaterial dispersion protocol for toxicological studies in ENPRA*, 6, ENPRA.

Javed, R., Ahmed, M., ul Haq, I., Nisa, S. and Zia, M. (2017) 'PVP and PEG doped CuO nanoparticles are more biologically active: Antibacterial, antioxidant, antidiabetic and cytotoxic perspective', *Materials Science & Engineering C-Materials for Biological Applications*, 79, 108-115.

Jiang, C., Castellon, B. T., Matson, C. W., Aiken, G. R. and Hsu-Kim, H. (2017) 'Relative contributions of copper oxide nanoparticles and dissolved copper to Cu uptake kinetics of gulf killifish (*Fundulus grandis*) embryos', *Environmental Science & Technology*, 51(3), 1395-1404.

Jiang, J., Oberdörster, G. and Biswas, P. (2009) 'Characterization of size, surface charge, and agglomeration state of nanoparticle dispersions for toxicological studies', *Journal of Nanoparticle Research*, 11(1), 77-89.

Ju-Nam, Y. and Lead, J. R. (2008) 'Manufactured nanoparticles: An overview of their chemistry, interactions and potential environmental implications', *Science of The Total Environment*, 400(1), 396-414.

Kehrer, J. P. (2000) 'The Haber–Weiss reaction and mechanisms of toxicity', *Toxicology*, 149(1), 43-50.

Keller, A. A., Adeleye, A. S., Conway, J. R., Garner, K. L., Zhao, L., Cherr, G. N., Hong, J., Gardea-Torresdey, J. L., Godwin, H. A., Hanna, S., Ji, Z., Kaweeteerawat, C., Lin, S., Lenihan, H. S., Miller, R. J., Nel, A. E., Peralta-Videa, J. R., Walker, S. L., Taylor, A. A., Torres-Duarte, C., Zink, J. I. and Zuverza-Mena, N. (2017) 'Comparative environmental fate and toxicity of copper nanomaterials', *NanoImpact*, 7(Supplement C), 28-40.

Keller, A. A. and Lazareva, A. (2014) 'Predicted releases of engineered nanomaterials: From global to regional to local', *Environmental Science & Technology Letters*, 1(1), 65-70.

Kent, R. D. and Vikesland, P. J. (2016) 'Dissolution and persistence of copper-based nanomaterials in undersaturated solutions with respect to cupric solid phases', *Environmental science & technology*, 50(13), 6772-6781.

Khomich, A. S., Axenov-Gribanov, D. V., Bodilovskaya, O. A., Shirokova, Y. A., Shchapova, E. P., Lubyaga, Y. A., Shatilina, Z. M., Emshanova, V. A. and Golubev, A. P. (2017) 'Assessment of the joint effect of thermal stress, pollution, and parasitic infestation on the activity of antioxidative enzymes in pulmonate mollusk *Lymnaea stagnalis*', *Contemporary Problems of Ecology*, 10(2), 157-163.

Kim, B.-M., Rhee, J.-S., Choi, I.-Y. and Lee, Y.-M. (2018) 'Transcriptional profiling of antioxidant defense system and heat shock protein (Hsp) families in the cadmium- and copper-exposed marine ciliate *Euplotes crassu*', *Genes & Genomics*, 40(1), 85-98.

Klaine, S. J., Alvarez, P. J. J., Batley, G. E., Fernandes, T. F., Handy, R. D., Lyon, D. Y., Mahendra, S., McLaughlin, M. J. and Lead, J. R. (2008) 'Nanomaterials in the environment: Behavior, fate, bioavailability, and effects', *Environmental Toxicology and Chemistry*, 27(9), 1825-1851.

Koene, J. M. and Ter Maat, A. (2004) 'Energy budgets in the simultaneously hermaphroditic pond snail, *Lymnaea stagnalis*: a trade-off between growth and reproduction during development', *Belgian Journal of Zoology*, 134(2/1), 41-46.

Koopman, K. R., Collas, F. P. L., van der Velde, G. and Verberk, W. C. E. P. (2016) 'Oxygen can limit heat tolerance in freshwater gastropods: differences between gill and lung breathers', *Hydrobiologia*, 763(1), 301-312.

Kovačec, E., Regvar, M., van Elteren, J. T., Arčon, I., Papp, T., Makovec, D. and Vogel-Mikuš, K. (2017) 'Biotransformation of copper oxide nanoparticles by the pathogenic fungus Botrytis cinerea', *Chemosphere*, 180, 178-185.

Kuhlbusch, T. and Nickel, C. (2010) *Nanoparticle emission of selected products during their life cycle* Dessau-Roßlau, Germany: Environmental Research of the Federal Ministry of the Environment, Nature Conservation and Nuclear Safety.

Kumar, A., Singh, S., Shanker, R. and Dhawan, A. (2018) 'Chapter 1-Nanotoxicology: Challenges for biologists' in *Nanotoxicology: Experimental and Computational Perspectives*, The Royal Society of Chemistry, 1-16.

Kumar, V., Kumari, A., Guleria, P. and Yadav, S. K. (2012) 'Evaluating the toxicity of selected types of nanochemicals' in *Reviews of Environmental Contamination and Toxicology*, New York, NY: Springer New York, 39-121.

Laurén, D. J. and McDonald, D. G. (1986) 'Influence of water hardness, pH, and alkalinity on the mechanisms of copper toxicity in juvenile rainbow trout, *Salmo gairdneri*', *Canadian Journal of Fisheries and Aquatic Sciences,* 43(8), 1488-1496.

Li, M., Zhu, L. and Lin, D. (2011) 'Toxicity of ZnO nanoparticles to *Escherichia coli*: Mechanism and the influence of medium components', *Environmental Science & Technology,* 45(5), 1977-1983.

Limbach, L. K., Wick, P., Manser, P., Grass, R. N., Bruinink, A. and Stark, W. J. (2007) 'Exposure of engineered nanoparticles to human lung epithelial cells: Influence of chemical composition and catalytic activity on oxidative stress', *Environmental Science & Technology,* 41(11), 4158-4163.

Lin, P.-C., Lin, S., Wang, P. C. and Sridhar, R. (2014) 'Techniques for physicochemical characterization of nanomaterials', *Biotechnology Advances,* 32(4), 711-726.

Liu, J., Fan, D., Wang, L., Shi, L., Ding, J., Chen, Y. and Shen, S. (2014) 'Effects of ZnO, CuO, Au, and TiO_2 nanoparticles on *Daphnia magna* and early life stages of zebrafish *Danio rerio*', *Environment Protection Engineering,* 40(1).

Louie, S. M., Tilton, R. D. and Lowry, G. V. (2016) 'Critical review: impacts of macromolecular coatings on critical physicochemical processes controlling environmental fate of nanomaterials', *Environmental Science-Nano,* 3(2), 283-310.

Lowry, G. V., Gregory, K. B., Apte, S. C. and Lead, J. R. (2012) 'Transformations of nanomaterials in the environment', *Environmental Science & Technology,* 46(13), 6893-6899.

Lukowiak, K. and Dalesman, S. (2013) 'Operant conditioning of respiration in *Lymnaea*: The environmental context' in *Handbook of Behavioral Neuroscience*, Elsevier, 265-279.

Lukowiak, K., Martens, K., Orr, M., Parvez, K., Rosenegger, D. and Sangha, S. (2006) 'Modulation of aerial respiratory behaviour in a pond snail', *Respiratory Physiology & Neurobiology,* 154(1), 61-72.

Lukowiak, K., Ringseis, E., Spencer, G., Wildering, W. and Syed, N. (1996) 'Operant conditioning of aerial respiratory behaviour in *Lymnaea stagnalis*', *Journal of Experimental Biology,* 199(3), 683-691.

Lukowiak, K., Sunada, H., Teskey, M. and Dalesman, S. (2014) 'Environmentally relevant stressors alter memory formation in the pond snail *Lymnaea*', *Journal of Experimental Biology,* 217(1), 76-83.

Lukowiak, K. and Syed, N. (1999) 'Learning, memory and a respiratory central pattern generator', *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* 124(3), 265-274.

Líbalová, H., Costa, P. M., Olsson, M., Farcal, L., Ortelli, S., Blosi, M., Topinka, J., Costa, A. L. and Fadeel, B. (2018) 'Toxicity of surface-modified copper oxide nanoparticles in a mouse macrophage cell line: Interplay of particles, surface coating and particle dissolution', *Chemosphere,* 196, 482-493.

Ma, T., Gong, S. and Tian, B. (2017) 'Effects of sediment-associated CuO nanoparticles on Cu bioaccumulation and oxidative stress responses in freshwater snail *Bellamya aeruginosa*', *Science of The Total Environment,* 580, 797-804.

Madhav, M. R., David, S. E. M., Kumar, R. S. S., Swathy, J. S., Bhuvaneshwari, M., Mukherjee, A. and Chandrasekaran, N. (2017) 'Toxicity and accumulation of copper oxide (CuO) nanoparticles in different life stages of *Artemia salina*', *Environmental Toxicology and Pharmacology*, 52, 227-238.

Malvern, I. L. 'Zeta potential - An introduction in 30 minutes', [online], available: https://www.malvern.com/en/support/resource-center/technical-notes/TN101104ZetaPotentialIntroduction].

Mansfield, E., Tyner, K. M., Poling, C. M. and Blacklock, J. L. (2014) 'Determination of nanoparticle surface coatings and nanoparticle purity using microscale thermogravimetric analysis', *Analytical chemistry*, 86(3), 1478-1484.

Marigómez, I., Soto, M., Cajaraville, M. P., Angulo, E. and Giamberini, L. (2002) 'Cellular and subcellular distribution of metals in molluscs', *Microscopy Research and Technique,* 56(5), 358-392.

Marques, M. R., Loebenberg, R. and Almukainzi, M. (2011) 'Simulated biological fluids with possible application in dissolution testing', *Dissolution Technol,* 18(3), 15-28.

Mazur, R., Shubiao, W., Szoszkiewicz, K., Bedla, D. and Nowak, A. (2016) 'A *Lymnaea stagnalis* embryo test for toxicity bioindication of acidification and ammonia pollution in water', *Water,* 8(7), 295.

Mazur, R., Wagner, A. and Zhou, M. (2013) 'The application of the *Lymnaea stagnalis* embryo-test in the toxicity bioindication of surfactants in fresh waters', *Ecological Indicators,* 30(0), 190-195.

McComb, C., Varshney, N. and Lukowiak, K. (2005) 'Juvenile *Lymnaea* ventilate, learn and remember differently than do adult *Lymnaea*', *Journal of Experimental Biology,* 208(8), 1459-1467.

Melegari, S. P., Perreault, F., Costa, R. H. R., Popovic, R. and Matias, W. G. (2013) 'Evaluation of toxicity and oxidative stress induced by copper oxide nanoparticles in the green alga *Chlamydomonas reinhardtii*', *Aquatic Toxicology,* 142–143, 431-440.

Miseljic, M. and Olsen, S. I. (2014) 'Life-cycle assessment of engineered nanomaterials: a literature review of assessment status', *Journal of Nanoparticle Research,* 16(6).

Misra, S. K., Dybowska, A., Berhanu, D., Croteau, M. N., Luoma, S. N., Boccaccini, A. R. and Valsami-Jones, E. (2012a) 'Isotopically modified nanoparticles for enhanced detection in bioaccumulation studies', *Environmental Science & Technology,* 46(2), 1216-1222.

Misra, S. K., Dybowska, A., Berhanu, D., Luoma, S. N. and Valsami-Jones, E. (2012b) 'The complexity of nanoparticle dissolution and its importance in nanotoxicological studies', *Science of The Total Environment,* 438, 225-232.

Mitrano, D. M., Motellier, S., Clavaguera, S. and Nowack, B. (2015) 'Review of nanomaterial aging and transformations through the life cycle of nano-enhanced products', *Environment International,* 77, 132-147.

Mitrano, D. M. and Nowack, B. (2017) 'The need for a life-cycle based aging paradigm for nanomaterials: importance of real-world test systems to identify realistic particle transformations', *Nanotechnology,* 28(7), 23.

Mohd Omar, F., Abdul Aziz, H. and Stoll, S. (2014) 'Aggregation and disaggregation of ZnO nanoparticles: Influence of pH and adsorption of Suwannee River humic acid', *Science of The Total Environment,* 468-469, 195-201.

Moore, J. D., Avellan, A., Noack, C. W., Guo, Y., Lowry, G. V. and Gregory, K. B. (2017) 'Time-dependent bacterial transcriptional response to CuO nanoparticles differs from that of Cu^{2+} and provides insights into CuO nanoparticle toxicity mechanisms', *Environmental Science: Nano*, 4(12), 2321-2335.

Moore, J. D., Stegemeier, J. P., Bibby, K., Marinakos, S. M., Lowry, G. V. and Gregory, K. B. (2016) 'Impacts of pristine and transformed Ag and Cu engineered nanomaterials on surficial sediment microbial communities appear short-lived', *Environmental Science & Technology*, 50(5), 2641-2651.

Mouneyrac, C., Buffet, P.-E., Poirier, L., Zalouk-Vergnoux, A., Guibbolini, M., Faverney, C. R.-d., Gilliland, D., Berhanu, D., Dybowska, A., Châtel, A., Perrein-Ettajni, H., Pan, J.-F., Thomas-Guyon, H., Reip, P. and Valsami-Jones, E. (2014) 'Fate and effects of metal-based nanoparticles in two marine invertebrates, the bivalve mollusc *Scrobicularia plana* and the annelid polychaete *Hediste diversicolor*', *Environmental Science and Pollution Research*, 21(13), 7899-7912.

Munley, K. M., Brix, K. V., Panlilio, J., Deforest, D. K. and Grosell, M. (2013) 'Growth inhibition in early life-stage tests predicts full life-cycle toxicity effects of lead in the freshwater pulmonate snail, Lymnaea stagnalis', *Aquatic Toxicology*, 128, 60-66.

Mwaanga, P., Carraway, E. R. and van den Hurk, P. (2014) 'The induction of biochemical changes in *Daphnia magna* by CuO and ZnO nanoparticles', *Aquatic Toxicology*, 150, 201-209.

Naatz, H., Lin, S., Li, R., Jiang, W., Ji, Z., Chang, C. H., Köser, J., Thöming, J., Xia, T., Nel, A. E., Mädler, L. and Pokhrel, S. (2017) 'Safe-by-design CuO nanoparticles via Fe-doping, Cu–O bond length variation, and biological assessment in cells and zebrafish embryos', *ACS Nano*, 11(1), 501-515.

Nagajyoti, P., Lee, K. and Sreekanth, T. (2010) 'Heavy metals, occurrence and toxicity for plants: a review', *Environmental Chemistry Letters*, 8(3), 199-216.

Nations, S., Long, M., Wages, M., Maul, J. D., Theodorakis, C. W. and Cobb, G. P. (2015) 'Subchronic and chronic developmental effects of copper oxide (CuO) nanoparticles on *Xenopus laevis*', *Chemosphere*, 135, 166-174.

Ng, T. Y. T., Pais, N. M. and Wood, C. M. (2011) 'Mechanisms of waterborne Cu toxicity to the pond snail *Lymnaea stagnalis*: Physiology and Cu bioavailability', *Ecotoxicology and Environmental Safety*, 74(6), 1471-1479.

Niyogi, S., Brix, K. V. and Grosell, M. (2014) 'Effects of chronic waterborne nickel exposure on growth, ion homeostasis, acid-base balance, arid nickel uptake in the freshwater pulmonate snail, Lymnaea stagnalis', *Aquatic Toxicology,* 150, 36-44.

Nowack, B., Boldrin, A., Caballero, A., Hansen, S. F., Gottschalk, F., Heggelund, L., Hennig, M., Mackevica, A., Maes, H., Navratilova, J., Neubauer, N., Peters, R., Rose, J., Schaffer, A., Scifo, L., van Leeuwen, S., von der Kammer, F., Wohlleben, W., Wyrwoll, A. and Hristozov, D. (2016) 'Meeting the Needs for Released Nanomaterials Required for Further Testing-The SUN Approach', *Environmental Science & Technology,* 50(6), 2747-2753.

Nowack, B., Brouwer, C., Geertsma, R. E., Heugens, E. H. W., Ross, B. L., Toufektsian, M.-C., Wijnhoven, S. W. P. and Aitken, R. J. (2012a) 'Analysis of the occupational, consumer and environmental exposure to engineered nanomaterials used in 10 technology sectors', *Nanotoxicology,* 7(6), 1152-1156.

Nowack, B., Ranville, J. F., Diamond, S., Gallego-Urrea, J. A., Metcalfe, C., Rose, J., Horne, N., Koelmans, A. A. and Klaine, S. J. (2012b) 'Potential scenarios for nanomaterial release and subsequent alteration in the environment', *Environmental Toxicology and Chemistry,* 31(1, SI), 50-59.

OECD (1992) 'Test No. 203: Fish, acute toxicity test',

OECD (2010) *List of manufactured nanomaterials and list of endpoints for phase one of the sponsorship programme for the testing of manufactured nanomaterials: Revision,* Organisation for Economic Co-operation and Development (OECD) Paris.

OECD (2016) 'Test No. 243: *Lymnaea stagnalis* reproduction test',

Oliver, A. L.-S., Croteau, M.-N., Stoiber, T. L., Tejamaya, M., Römer, I., Lead, J. R. and Luoma, S. N. (2014) 'Does water chemistry affect the dietary uptake and toxicity of silver nanoparticles by the freshwater snail *Lymnaea stagnalis*?', *Environmental Pollution,* 189(Supplement C), 87-91.

Orr, M. V., El-Bekai, M., Lui, M., Watson, K. and Lukowiak, K. (2007) 'Predator detection in *Lymnaea stagnalis*', *Journal of Experimental Biology,* 210(23), 4150-4158.

Orr, M. V. and Lukowiak, K. (2008) 'Electrophysiological and behavioral evidence demonstrating that predator detection alters adaptive behaviors in the snail *Lymnaea*', *Journal of Neuroscience,* 28(11), 2726-2734.

Ortelli, S., Costa, A. L., Blosi, M., Brunelli, A., Badetti, E., Bonetto, A., Hristozov, D. and Marcomini, A. (2017) 'Colloidal characterization of CuO nanoparticles in biological and environmental media', *Environmental Science: Nano*.

O'Gara, B. A., Bohannon, V. K., Teague, M. W. and Smeaton, M. B. (2004) 'Copper-induced changes in locomotor behaviors and neuronal physiology of the freshwater oligochaete, *Lumbriculus variegatus*', *Aquatic Toxicology,* 69(1), 51-66.

Pang, C., Selck, H., Misra, S. K., Berhanu, D., Dybowska, A., Valsami-Jones, E. and Forbes, V. E. (2012) 'Effects of sediment-associated copper to the deposit-feeding snail, *Potamopyrgus antipodarum*: a comparison of Cu added in aqueous form or as nano-and micro-CuO particles', *Aquatic toxicology,* 106, 114-122.

Park, E.-J., Yi, J., Kim, Y., Choi, K. and Park, K. (2010) 'Silver nanoparticles induce cytotoxicity by a Trojan-horse type mechanism', *Toxicology in Vitro,* 24(3), 872-878.

Park, J.-W., Hecker, M., Murphy, M. B., Jones, P. D., Solomon, K. R., Van Der Kraak, G., Carr, J. A., Smith, E. E., du Preez, L., Kendall, R. J. and Giesy, J. P. (2006) 'Development and optimization of a Q-RT PCR method to quantify CYP19 mRNA expression in testis of male adult *Xenopus laevis*: Comparisons with aromatase enzyme activity', *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* 144(1), 18-28.

Parvez, K., Rosenegger, D., Orr, M., Martens, K. and Lukowiak, K. (2006) 'Canadian Association of Neurosciences Review: learning at a snail's pace', *Canadian Journal of Neurological Sciences,* 33(4), 347-356.

Parvez, K., Stewart, O., Sangha, S. and Lukowiak, K. (2005) 'Boosting intermediate-term into long-term memory', *Journal of Experimental Biology,* 208(8), 1525-1536.

Peijnenburg, W. J. G. M., Baalousha, M., Chen, J., Chaudry, Q., Von der kammer, F., Kuhlbusch, T. A. J., Lead, J., Nickel, C., Quik, J. T. K., Renker, M., Wang, Z. and Koelmans, A. A. (2015) 'A review of the properties and processes determining the fate of engineered nanomaterials in the aquatic environment', *Critical Reviews in Environmental Science and Technology,* 45(19), 2084-2134.

Peng, C., Shen, C., Zheng, S., Yang, W., Hu, H., Liu, J. and Shi, J. (2017) 'Transformation of CuO nanoparticles in the aquatic environment: Influence of pH, electrolytes and natural organic matter', *Nanomaterials,* 7(10), 326.

Perreault, F., Oukarroum, A., Melegari, S. P., Matias, W. G. and Popovic, R. (2012) 'Polymer coating of copper oxide nanoparticles increases nanoparticles uptake and toxicity in the green alga *Chlamydomonas reinhardtii*', *Chemosphere*, 87(11), 1388-1394.

Pfaffl, M. W. (2001) 'A new mathematical model for relative quantification in real-time RT–PCR', *Nucleic Acids Research*, 29(9), e45-e45.

Platten, W. E., Sylvest, N., Warren, C., Arambewela, M., Harmon, S., Bradham, K., Rogers, K., Thomas, T. and Luxton, T. P. (2016) 'Estimating dermal transfer of copper particles from the surfaces of pressure-treated lumber and implications for exposure', *Science of The Total Environment*, 548-549, 441-449.

Podolski, I. Y., Kondratjeva, E. V., Gurin, S. S., Dumpis, M. A. and Piotrovsky, L. B. (2005) 'Fullerene C_{60} complexed with poly(n-vinyl-pyrrolidone) (C_{60}/PVP) prevents the disturbance of long-term memory consolidation induced by cycloheximide', *Fullerenes, Nanotubes and Carbon Nanostructures*, 12(1-2), 421-424.

Prabhu, B. M., Ali, S. F., Murdock, R. C., Hussain, S. M. and Srivatsan, M. (2010) 'Copper nanoparticles exert size and concentration dependent toxicity on somatosensory neurons of rat', *Nanotoxicology*, 4(2), 150-160.

Pradhan, A., Geraldes, P., Seena, S., Pascoal, C. and Cássio, F. (2015) 'Natural organic matter alters size-dependent effects of nanoCuO on the feeding behaviour of freshwater invertebrate shredders', *Science of The Total Environment*, 535, 94-101.

Pradhan, A., Seena, S., Pascoal, C. and Cassio, F. (2012) 'Copper oxide nanoparticles can induce toxicity to the freshwater shredder *Allogamus ligonifer*', *Chemosphere*, 89(9), 1142-50.

Pradhan, A., Silva, C. O., Silva, C., Pascoal, C. and Cássio, F. (2016) 'Enzymatic biomarkers can portray nanoCuO-induced oxidative and neuronal stress in freshwater shredders', *Aquatic Toxicology*, 180, 227-235.

Pu, Y., Tang, F., Adam, P.-M., Laratte, B. and Ionescu, R. E. (2016) 'Fate and characterization factors of nanoparticles in seventeen subcontinental freshwaters: A sase study on copper nanoparticles', *Environmental Science & Technology*, 50(17), 9370-9379.

Ramskov, T., Croteau, M.-N., Forbes, V. E. and Selck, H. (2015) 'Biokinetics of different-shaped copper oxide nanoparticles in the freshwater gastropod, *Potamopyrgus antipodarum*', *Aquatic Toxicology*, 163(Supplement C), 71-80.

Ramskov, T., Selck, H., Banta, G., Misra, S. K., Berhanu, D., Valsami-Jones, E. and Forbes, V. E. (2014) 'Bioaccumulation and effects of different-shaped copper oxide nanoparticles in the deposit-feeding snail *Potamopyrgus antipodarum*', *Environmental Toxicology and Chemistry*, 33(9), 1976-1987.

Rasband, W. S. (2011) 'Imagej, us national institutes of health, bethesda, maryland, usa', *http://imagej. nih. gov/ij/*.

Rauscher, H., Rasmussen, K. and Sokull-Klüttgen, B. (2017) 'Regulatory aspects of nanomaterials in the EU', *Chemie Ingenieur Technik*, 89(3), 224-231.

Rauscher, H., Roebben, G., Amenta, V., Boix, S., Calzolai, L., Emons, H., Gaillard, C., Gibson, P., Linsinger, T. and Mech, A. (2014) *Towards a review of the EC Recommendation for a definition of the term "nanomaterial" Part 1: Compilation of information concerning the experience with the definition*, 26567.

Rauscher, H., Roebben, G., Boix Sanfeliu, A., Emons, H., Gibson, N., Koeber, R., Linsinger, T., Rasmussen, K., Riego Sintes, J. and Sokull-Kltgen, B. (2015) *Towards a review of the EC recommendation for a definition of the term ''nanomaterial'' Part 3: scientific-technical evaluation of options to clarify the definition and to facilitate its implementation*, 27240.

Regoli, F., Benedetti, M. and Giuliani, M. E. (2011) 'Antioxidant defenses and acquisition of tolerance to chemical stress', *Tolerance to Environmental Contaminants*, 153-173.

Rippner, D. A., Green, P. G., Young, T. M. and Parikh, S. J. (2018) 'Dissolved organic matter reduces CuO nanoparticle toxicity to duckweed in simulated natural systems', *Environmental Pollution*, 234, 692-698.

Rogevich, E. C., Hoang, T. C. and Rand, G. M. (2009) 'Effects of sublethal chronic copper exposure on the growth and reproductive success of the florida apple snail (*Pomacea paludosa*)', *Archives of Environmental Contamination and Toxicology*, 56(3), 450-458.

Rosenegger, D., Parvez, K. and Lukowiak, K. (2008) 'Enhancing memory formation by altering protein phosphorylation balance', *Neurobiology of Learning and Memory*, 90(3), 544-552.

Rossetto, A. L. d. O. F., Melegari, S. P., Ouriques, L. C. and Matias, W. G. (2014) 'Comparative evaluation of acute and chronic toxicities of CuO nanoparticles and bulk using Daphnia magna and Vibrio fischeri', *Science of The Total Environment*, 490(Supplement C), 807-814.

Ruppert, E. E., Barnes, R. D. and Fox, R. S. (2004) *Invertebrate zoology: a functional evolutionary approach*.

Salo, T., Stamm, C., Burdon Francis, J., Räsänen, K. and Seppälä, O. (2017) 'Resilience to heat waves in the aquatic snail *Lymnaea stagnalis*: Additive and interactive effects with micropollutants', *Freshwater Biology*, 62(11), 1831-1846.

Santore, R. C., Di Toro, D. M., Paquin, P. R., Allen, H. E. and Meyer, J. S. (2001) 'Biotic ligand model of the acute toxicity of metals. 2. Application to acute copper toxicity in freshwater fish and *Daphnia*', *Environmental Toxicology and Chemistry*, 20(10), 2397-2402.

Schlekat, C. E., Van Genderen, E., De Schamphelaere, K. A. C., Antunes, P. M. C., Rogevich, E. C. and Stubblefield, W. A. (2010) 'Cross-species extrapolation of chronic nickel biotic ligand models', *Science of the Total Environment*, 408(24), 6148-6157.

Schwarz-Plaschg, C., Kallhoff, A. and Eisenberger, I. (2017) 'Making nanomaterials safer by design?', *NanoEthics*, 11(3), 277-281.

Scott-Fordsmand, J., Peijnenburg, W., Semenzin, E., Nowack, B., Hunt, N., Hristozov, D., Marcomini, A., Irfan, M. A., Jiménez, A. S., Landsiedel, R., Tran, L., Oomen, A., Bos, P. and Hund-Rinke, K. (2017) 'Environmental risk assessment strategy for nanomaterials', *International Journal of Environmental Research and Public Health*, 14(10), 1251.

Sharifi, S., Behzadi, S., Laurent, S., Laird Forrest, M., Stroeve, P. and Mahmoudi, M. (2012) 'Toxicity of nanomaterials', *Chemical Society Reviews*, 41(6), 2323-2343.

Sharma, H. S. and Sharma, A. (2007) 'Nanoparticles aggravate heat stress induced cognitive deficits, blood–brain barrier disruption, edema formation and brain pathology' in Sharma, H. S., ed. *Progress in Brain Research*, Elsevier, 245-273.

Shi, J. Y., Abid, A. D., Kennedy, I. M., Hristova, K. R. and Silk, W. K. (2011) 'To duckweeds (*Landoltia punctata*), nanoparticulate copper oxide is more inhibitory than the soluble copper in the bulk solution', *Environmental Pollution*, 159(5), 1277-1282.

Siddiqui, S., Goddard, R. H. and Bielmyer-Fraser, G. K. (2015) 'Comparative effects of dissolved copper and copper oxide nanoparticle exposure to the sea anemone, *Exaiptasia pallida*', *Aquatic Toxicology*, 160(Supplement C), 205-213.

Sidorov, A. V. (2005) 'Effect of acute temperature change on lung respiration of the mollusc *Lymnaea stagnalis*', *Journal of Thermal Biology*, 30(2), 163-171.

Simkiss, K. and Mason, A. Z. (1983) 'Metal ions: Metabolic and toxic effects' in Hochachka, P. W., ed. *The Mollusca*, San Diego: Academic Press, 101-164.

Singh, J., Kaur, G. and Rawat, M. (2016) 'A brief review on synthesis and characterization of copper oxide nanoparticles and its applications', *Journal of Bioelectronics and Nanotechnology,* 1(1), 9.

Skinner, B. F. (1965) *Science And Human Behavior*, Free Press.

Socci, D. J., Crandall, B. M. and Arendash, G. W. (1995) 'Chronic antioxidant treatment improves the cognitive performance of aged rats', *Brain Research,* 693(1), 88-94.

Soni, D., Naoghare, P. K., Saravanadevi, S. and Pandey, R. A. (2015) 'Release, transport and toxicity of engineered nanoparticles' in Whitacre, D. M., ed. *Reviews of Environmental Contamination and Toxicology*, Cham: Springer International Publishing, 1-47.

Sousa, V. S. and Teixeira, M. R. (2013) 'Aggregation kinetics and surface charge of CuO nanoparticles: the influence of pH, ionic strength and humic acids', *Environmental Chemistry,* 10(4), 313-322.

Sovová, T., Boyle, D., Sloman, K. A., Vanegas Pérez, C. and Handy, R. D. (2014) 'Impaired behavioural response to alarm substance in rainbow trout exposed to copper nanoparticles', *Aquatic Toxicology,* 152, 195-204.

Stansley, B. J. and Yamamoto, B. K. (2015) 'Behavioral impairments and serotonin reductions in rats after chronic L-dopa', *Psychopharmacology,* 232(17), 3203-3213.

Stegemeier, J. P., Avellan, A. and Lowry, G. V. (2017) 'Effect of initial speciation of copper and silver-based nanoparticles on their long-term fate and phytoavailability in freshwater wetland Mmesocosms', *Environmental Science & Technology,* 51(21), 12114-12122.

Stoiber, T., Croteau, M.-N., Römer, I., Tejamaya, M., Lead, J. R. and Luoma, S. N. (2015) 'Influence of hardness on the bioavailability of silver to a freshwater snail after waterborne exposure to silver nitrate and silver nanoparticles', *Nanotoxicology,* 9(7), 918-927.

Strong, C. R. and Luoma, S. N. (1981) 'Variations in the correlation of body size with concentrations of Cu and Ag in the bivalve *Macoma balthica*', *Canadian Journal of Fisheries and Aquatic Sciences,* 38(9), 1059-1064.

Sugai, R., Shiga, H., Azami, S., Watanabe, T., Sadamoto, H., Fujito, Y., Lukowiak, K. and Ito, E. (2006) 'Taste discrimination in conditioned taste aversion of the pond snail *Lymnaea stagnalis*', *Journal of Experimental Biology,* 209(5), 826-833.

SUN (2014a) *Deliverable 1.4: Report on characterization of pristine nanomaterials for (eco)toxicological testing*.

SUN (2014b) *Milestone 6: Delivery of release NOAA for testing in other WPs*.

SUN (2015) *Deliverable 7.1: Report on the development of SbyD strategies applied to CuO*.

Sun, T. Y., Bornhöft, N. A., Hungerbühler, K. and Nowack, B. (2016) 'Dynamic probabilistic modeling of environmental emissions of engineered nanomaterials', *Environmental Science & Technology,* 50(9), 4701-4711.

Sunada, H., Riaz, H., de Freitas, E., Lukowiak, K., Swinton, C., Swinton, E., Protheroe, A., Shymansky, T., Komatsuzaki, Y. and Lukowiak, K. (2016) 'Heat stress enhances LTM formation in *Lymnaea*: role of HSPs and DNA methylation', *Journal of Experimental Biology,* 219(9), 1337-1345.

Sureda, A., Capó, X., Busquets-Cortés, C. and Tejada, S. (2018) 'Acute exposure to sunscreen containing titanium induces an adaptive response and oxidative stress in *Mytillus galloprovincialis*', *Ecotoxicology and environmental safety,* 149, 58-63.

Takigami, S., Sunada, H., Lukowiak, K., Kuzirian, A. M., Alkon, D. L. and Sakakibara, M. (2014) 'Protein kinase C mediates memory consolidation of taste avoidance conditioning in Lymnaea stagnalis', *Neurobiology of Learning and Memory,* 111, 9-18.

Taylor, B. E., Harris, M. B., Burk, M., Smyth, K., Lukowiak, K. and Remmers, J. E. (2003) 'Nitric oxide mediates metabolism as well as respiratory and cardiac responses to hypoxia in the snail *Lymnaea stagnalis*', *Journal of Experimental Zoology Part A: Comparative Experimental Biology,* 295A(1), 37-46.

Taylor, H. H. and Anstiss, J. M. (1999) 'Copper and haemocyanin dynamics in aquatic invertebrates', *Marine and Freshwater Research,* 50(8), 907-931.

Tchounwou, P. B., Yedjou, C. G., Patlolla, A. K. and Sutton, D. J. (2012) 'Heavy metal toxicity and the environment' in Luch, A., ed. *Molecular, Clinical and Environmental Toxicology: Volume 3: Environmental Toxicology*, Basel: Springer Basel, 133-164.

Tejamaya, M., Römer, I., Merrifield, R. C. and Lead, J. R. (2012) 'Stability of citrate, PVP, and PEG coated silver nanoparticles in ecotoxicology media', *Environmental Science & Technology,* 46(13), 7011-7017.

Ter Maat, A., Pieneman, A. and Koene, J. (2012) 'The effect of light on induced egg laying in the simultaneous hermaphrodite *Lymnaea stagnalis*', *Journal of Molluscan Studies,* 78(3), 262-267.

Ter Maat, A., Zonneveld, C., de Visser, J. A. G., Jansen, R. F., Montagne-Wajer, K. and Koene, J. M. (2007) 'Food intake, growth, and reproduction as affected by day length and food availability in the pond snail *Lymnaea stagnalis*', *American Malacological Bulletin,* 23(1), 113-120.

Teskey, M. L., Lukowiak, K. S., Riaz, H., Dalesman, S. and Lukowiak, K. (2012) 'What's hot: the enhancing effects of thermal stress on long-term memory formation in *Lymnaea stagnalis*', *Journal of Experimental Biology,* 215(24), 4322-4329.

Torres-Duarte, C., Adeleye, A. S., Pokhrel, S., Mädler, L., Keller, A. A. and Cherr, G. N. (2016) 'Developmental effects of two different copper oxide nanomaterials in sea urchin (*Lytechinus pictus*) embryos', *Nanotoxicology,* 10(6), 671-679.

Turner, A. M., Fetterolf, S. A. and Bernot, R. J. (1999) 'Predator identity and consumer behavior: differential effects of fish and crayfish on the habitat use of a freshwater snail', *Oecologia,* 118(2), 242-247.

van Pomeren, M., Brun, N. R., Peijnenburg, W. J. G. M. and Vijver, M. G. (2017) 'Exploring uptake and biodistribution of polystyrene (nano)particles in zebrafish embryos at different developmental stages', *Aquatic Toxicology,* 190, 40-45.

Vencalek, B. E., Laughton, S. N., Spielman-Sun, E., Rodrigues, S. M., Unrine, J. M., Lowry, G. V. and Gregory, K. B. (2016) '*In situ* measurement of CuO and $Cu(OH)_2$ nanoparticle dissolution rates in quiescent freshwater mesocosms', *Environmental Science & Technology Letters,* 3(10), 375-380.

Villarreal, F. D., Das, G. K., Abid, A., Kennedy, I. M. and Kultz, D. (2014) 'Sublethal effects of CuO nanoparticles on mozambique tilapia (*Oreochromis mossambicus*) are modulated by environmental salinity', *PlosOne,* 9(2), 15.

Wahab, R., Khan, S. T., Dwivedi, S., Ahamed, M., Musarrat, J. and Al-Khedhairy, A. A. (2013) 'Effective inhibition of bacterial respiration and growth by CuO microspheres composed of thin nanosheets', *Colloids and Surfaces B: Biointerfaces,* 111, 211-217.

Wang, H., Fan, W., Xue, F., Wang, X., Li, X. and Guo, L. (2015) 'Chronic effects of six micro/nano-Cu_2O crystals with different structures and shapes on *Daphnia magna*', *Environmental Pollution,* 203, 60-68.

Wang, Z., Von Dem Bussche, A., Kabadi, P. K., Kane, A. B. and Hurt, R. H. (2013) 'Biological and environmental transformations of copper-based nanomaterials'.

Woo, S., Denis, V., Won, H., Shin, K., Lee, G., Lee, T.-K. and Yum, S. (2013) 'Expressions of oxidative stress-related genes and antioxidant enzyme activities in *Mytilus galloprovincialis* (Bivalvia, Mollusca) exposed to hypoxia', *Zoological Studies,* 52(1), 15.

Wu, F., Bortvedt, A., Harper, B. J., Crandon, L. E. and Harper, S. L. (2017) 'Uptake and toxicity of CuO nanoparticles to *Daphnia magna* varies between indirect dietary and direct waterborne exposures', *Aquatic Toxicology,* 190(Supplement C), 78-86.

Xiao, Y., Vijver, M. G. and Peijnenburg, W. J. G. M. (2018) 'Impact of water chemistry on the behavior and fate of copper nanoparticles', *Environmental Pollution,* 234, 684-691.

Young, A., Protheroe, A. and Lukowiak, K. (2017) 'Silver nanoparticles alter learning and memory formation in an aquatic organism, Lymnaea stagnalis', *Environmental Pollution,* 225(Supplement C), 403-411.

Young, K. G., Chang, J. P. and Goldberg, J. I. (1999) 'Gonadotropin-releasing hormone neuronal system of the freshwater snails *Helisoma trivolvis* and *Lymnaea stagnalis*: Possible involvement in reproduction', *Journal of comparative neurology,* 404(4), 427-437.

Zaporotskova, I. V. and Chernozatonskii, L. A. (2005) 'A study on the mechanism of interaction between fullerene and cycloheximide for the explanation of the beneficial effect of C_{60} on the processes of spatial memory restoration', *Mendeleev Communications,* 15(6), 227-229.

Zevenhuizen, L. P. T. M., Dolfing, J., Eshuis, E. J. and Scholten-Koerselman, I. J. (1979) 'Inhibitory effects of copper on bacteria related to the free ion concentration', *Microbial Ecology,* 5(2), 139-146.

Zhang, D. Q., Hua, T., Xiao, F., Chen, C. P., Gersberg, R. M., Liu, Y., Ng, W. J. and Tan, S. K. (2014) 'Uptake and accumulation of CuO nanoparticles and CdS/ZnS quantum dot nanoparticles by *Schoenoplectus tabernaemontani* in hydroponic mesocosms', *Ecological Engineering,* 70, 114-123.

Zhao, L., Hu, Q., Huang, Y., Fulton, A. N., Hannah-Bick, C., Adeleye, A. S. and Keller, A. A. (2017) 'Activation of antioxidant and detoxification gene expression in cucumber plants exposed to a $Cu(OH)_2$ nanopesticide', *Environmental Science: Nano*, 4(8), 1750-1760.

Zhao, L., Ortiz, C., Adeleye, A. S., Hu, Q., Zhou, H., Huang, Y. and Keller, A. A. (2016) 'Metabolomics to detect response of lettuce (*Lactuca sativa*) to $Cu(OH)_2$ nanopesticides: Oxidative stress response and detoxification mechanisms', *Environmental Science & Technology*, 50(17), 9697-9707.